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THE EFFECT OF TEMPERATURE ON THE INTERSEX CHARACTER OF DAPHNIA LONGISPINA[1]

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Sex intergrades, usually females with certain male secondary sex characters, have been observed in several species of cladocerans or water fleas (Banta, 1918; De la Vaulx, 1922). These sex intergrades appear to be genetic mutants, for, in the cases reported, the intersex appeared in a clone of normal animals and transmitted the intersex character to her descendants. Among the descendants, however, which were produced parthenogenetically, manifestation of the character was variable, with the full gamut of intersex expression from normal females to extreme sex intergrades occurring even within the same brood of young. In diploid parthenogenesis all offspring should be genetically alike, barring spontaneous mutation, since propagation from a single individual rules out the admixture and recombination of genetic factors that occur in sexual reproduction. The observed variability of expression among animals which should be genetically homogeneous suggests that the processes concerned in the development of the intersex character may be sensitive to environmental conditions. In view of a study by Wood (1939) on sex intergrades in *Daphnia longispina* in which she incidentally noted some effect of culture temperature on the manifestation of the intersex character, the following experiments were carried out to test the effect of temperature on intersex expression in *D. longispina*.

TEMPERATURE AND MANIFESTATION OF INTERSEXUALITY

The intersex clone used in these experiments was Banta's line 1612 (Banta *et al.*, 1939), which had been cultured in the laboratory solely by diploid parthenogenesis for over ten years. In this line the intersex animals are females with male-like secondary sex characters. Gonads with few exceptions appear to be unaltered; only rarely has a male sex intergrade been reported (Banta *et al.*, 1939).

There are five important secondary sex characters recognized in *D. longispina* (Figs. 1 and 2): (1) the body size of the female is considerably greater than that of the male; (2) the female beak is

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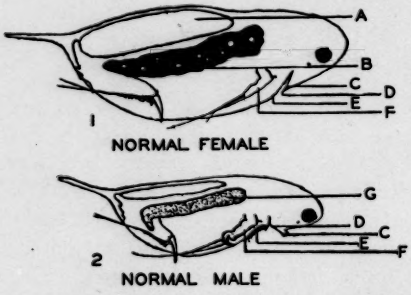
pointed, while that of the male is much shortened and rounded; (3) the antennule of each side in the female is represented only by a minute eminence bearing several fine sensory filaments, whereas in the male the antennules are elongate, rodlike, movable structures, projecting slightly forward and bearing a spine and sensory filaments on the anterior and posterior portions, respectively, of the distal end; (4) the anterior, ventral margins of the carapace or breast



FIGS. 1 and 2.—Normal female and male *Daphnia longispina*. *A*, brood chamber; *B*, ovary; *C*, antennule; *D*, beak; *E*, breast margin; *F*, appendage; *G*, testis.

margins of the female are uniformly rounded and lack hairs, while in the male the margins are angular, with a hair-fringed excavation in the anterior ventral region; and (5) the female first appendage bears two slender, tapering filaments, one long and one short, while the male first leg is characterized by the presence of a filament heavier and longer than those of the female, a very short secondary filament, and a stout chitinized hook.

In the sex intergrades, modifications of varying extent have been observed in all these secondary sex characters. The antennule region of the female may be slightly enlarged, bear a spine often abnormally long, be extended into a minute antennule with or without a spine, or be represented by one or two true male-like antennules of varying lengths. Intermediate conditions of body size, beak, and breast-margin contour and fine gradations of intermediacy in first-leg structure likewise occur.

A four-category system of grading was devised with classification of the females as "normal" or as "slight," "intermediate," or "extreme" in their expression of maleness. "Normal" females were, of course, not intersexes. The "slight" category (Figs. 3 and 4) included all animals showing any male influence in beak or antennule, since these are the characters most readily modified, and the few cases

FIGS. 3–6.—*A*, antennule; *B*, beak; *C*, breast margin; *D*, first leg. Fig. 3, a very slightly intergrade female with beak less pointed than in the normal female. The antennule region exhibits a tiny spine, suggesting male influence. The first legs and breast margins are unmodified. Fig. 4, a slightly intergrade female exhibiting a somewhat rounded beak and male-like antennules. The breast margins and first legs are female in character. Fig. 5, an intermediate sex intergrade female with male-like beak and antennules. The breast margin is slightly angular, bearing a few hairs. One first leg shows a male-like hook. Fig. 6, an extremely intergrade female, exhibiting the full complement of male secondary sex characters.

in which beak and antennule were unaltered but some male-like modification occurred in one of the other secondary sex characters. The "intermediate" group (Fig. 5) included animals with marked expression of maleness in antennules and beak and at least one first appendage. Among the sex intergrades of *D. longispina*, greater male influence is frequently observable in one antennule or one first appendage than in the other. The "extreme" animals (Fig. 6) exhibited male influence in all secondary sex characters, to the extent of appearing almost identical with males, except for their slightly larger body size and possession of ovaries instead of testes.

The grade assigned was based on a microscopic study under low-power objective of sexually mature animals in their adolescent, first adult, or, occasionally, second adult instar. The adolescent (fourth) instar marks the beginning of functional activity of the ovaries. At the beginning of the first adult (fifth)instar, a clutch of eggs from the ovaries passes through the two oviducts into the dorsal brood chamber, where embryonic development takes place. This instar terminates with the molt, following release of the first clutch of young from the brood chamber. In each of the subsequent adult instars until senescence is pronounced, a brood of young is normally produced.

The culture medium used for the stock cultures and during the testing was Banta's manure infusion (1921), slightly modified in that 2 quarts of horse manure and one quart of garden soil were soaked in pond water. This concentrated infusion was diluted approximately 1:8 with pond water.[3]

[3] Considerable effort was made to secure a more uniform and standardized medium, in which the food supply would be less likely to be altered by temperature than in the medium described above. When, in an effort to eliminate any secondary temperature effect in these experiments, bacteria-free *Daphnia* were cultured either in sterile pond water or in sterile synthetic pond water to which definite quantities of slow-growing unicellular algae had been added, the reduction in numbers of offspring indicated an inadequate food supply, and, consequently, these types of media were judged impracticable. Furthermore, subsequent experiments localizing the temperature-action effective period, as described below, appeared to rule out food supply as a major influence in altering intersex expression.

In a preliminary set of experiments, only two temperatures were used, 25° and 15° C.; but subsequently a third test temperature was introduced, 29° C., although lowered reproductive capacity was anticipated and did occur. At the lowest temperature an instar lasted from 4 to 5 days as compared with the 48–24-hour instar periods at 25° and 29° C. In each series young sibs were placed, one per bottle, at each temperature level to serve at maturity as the mothers of test clutches of offspring. As soon as young were released, the mother and all the young were transferred to fresh media of the requisite temperature, the mother to one bottle and three or four young to each additional bottle. This procedure was followed at the release of each subsequent brood of a mother. The offspring were cultured at controlled temperatures until time for grading at sexual maturity.

The results of nine series of experiments are summarized in Table 1. Data for the low-temperature stock in Series VII and for animals cultured at 29° C. in Series IX are lacking because of a high mortality rate. The table shows the number of test mothers at each temperature (third col.), the numbers of offspring produced (fourth col.), the number of offspring graded (fifth col.), the number of male offspring (sixth col.), the percentages of normal, slight, intermediate, and extreme intersex offspring (seventh to tenth cols.), and the average grade of the intersexes (eleventh and

twelfth cols.).[4] In determining the average grade of the offspring, a numerical grading system was used in which a value of 0 was assigned to normal-appearing females and 1, 2, and 3 to those with "slight," "intermediate," and "extreme" forms of intersex expression. To obtain the average value, the sum of the number of animals in each category multiplied by the value assigned that grade was divided by the total number of animals described. In view of the sporadic nature of male production and the apparent lack of correlation with intersex expression, the number of males was not used in the computation of the numerical average,

[4] The disparity between the number of offspring produced and described is due primarily to mortality of the offspring before sexual maturity, much of which resulted from the extremely early transfer of young animals to fresh media.

TABLE 1

EFFECT OF TEMPERATURE ON THE INTERSEX CHARACTER OF *Daphnia longispina*

Series	No. of Mothers	No. of Offspring	No. of Offspring Graded	No. of Males	Intersex Expression				Grade of Offspring	
					% Normal	% Slight	% Intermediate	% Extreme	Average for Series	Average for Culture Temperature
				29° C.						
III	5	291	188	0	89	11	0	0	0.11	0.08
IV	6	408	241	0	95	5	0	0	0.05	
V	1	46	29	5	79	21	0	0	0.21	
VI	4	257	199	0	90	10	0	0	0.10	
VII	2	117	91	0	98	2	0	0	0.02	
VIII	2	124	104	1	90	10	0	0	0.10	
				25° C.						
I	4	403	299	1	71	28	1	0	0.31	0.29
II	7	957	750	41	75	24	1	0	0.25	
III	3	507	378	18	68	31	2	0	0.34	
IV	6	873	618	55	71	26	2	1	0.31	
V	1	137	97	13	79	21	0	0	0.21	
VI	4	346	301	21	63	33	2	0	0.38	
VII	3	398	301	22	78	22	0	0	0.23	
VIII	2	192	149	11	83	17	0	0	0.17	
IX	2	215	196	22	70	29	2	0	0.32	
				15° C.						
I	5	574	520	44	15	72	12	1	0.99	1.02
II	5	624	585	60	15	76	9	1	0.96	
III	2	260	231	0	6	57	23	14	1.45	
IV	2	153	100	6	27	49	13	12	1.10	
V	2	273	244	1	3	68	23	6	1.32	
VI	1	97	92	0	12	72	15	1	1.05	
VIII	4	851	773	2	17	73	9	0	0.93	
IX	4	526	471	50	20	70	9	1	0.90	

which therefore serves as a quantitative measure of the degree of intersex expression.

The data as summarized in Table 1 indicate clearly a definite effect of temperature on intersex expression, culture temperatures of 29°, 25°, and 15° C. producing average grades of 0.08, 0.29, and 1.02. Incidental observation of over a hundred offspring of mothers cultured at 32° C., where mortality is too high to yield large numbers of young, revealed

ment, these test mothers were subjected, usually after an interval of two instars at one temperature, to an abrupt change to the other temperature, directly after a recording of the stage of egg development in the ovary or in the brood chamber. Their offspring thus fell into three categories: (1) those that had undergone complete ovarian, embryonic, and postnatal development at one temperature, (2) those that had been transferred from one temperature to another during the

TABLE 2

FIRST TRANSFER SERIES

Temperature Treatment	No. of Broods	No. of Offspring	No. of Offspring Described	No. of Males	Intersex expression				Average Grade of Offspring
					% Normal	% Slight	% Intermediate	% Extreme	
Complete development at 25° C.	138	1,807	1,450	59	43	56	1	0	0.58
Transfers from 25° to 12° C. during embr. dev.	62	1,126	986	39	13	86	1	0	0.88
Transfers from 25° to 12° C. during ovarian dev.	28	592	557	1	6	86	7	1	1.04
Complete development at 12° C.	39	607	459	53	4	87	7	2	1.07
Transfers from 12° to 25° C. during emb. dev.	62	1,040	527	136	13	63	15	9	1.21
Transfers from 12° to 25° C. during ovarian dev.	38	631	444	13	30	69	1	0	0.71

complete inhibition of intersex expression at this maximum temperature.

THE TEMPERATURE-EFFECTIVE PERIOD

With the establishment of a relationship between intersex expression and temperature of culture, two series of experiments were carried out to determine whether or not there is a short, well-defined temperature-effective period.

In the first series, animals in their second or third instar were selected from the low-temperature stock used in the previous experiments and were placed, one per bottle, at 25° and 12° C., to serve as the mothers of the animals to be graded. During the course of the experi-

ovarian period, and (3) those that had been transferred from one temperature to another during early embryonic development. The results of this series of experiments are summarized in Table 2, in which an additional column, the number of broods of offspring subjected to each temperature treatment, is also included.

The most striking feature of the data from this series of experiments is the very evident dependence of intersex expression on the length of exposure to low temperature during culture. This is reflected in all averages, with the exception of the relative values for transfers from low to high temperatures during embryonic development, as compared with

that for animals undergoing complete development at 12° C.

The second set of transfer experiments differed from the first in that greater attention was paid to the temperature history of the test mothers to insure as great homogeneity as possible in the biological material of the test. Offspring of one mother were used as the test mothers. The mother exhibiting a very slight grade of intersex expression had undergone complete development, ovarian, embryonic, and postnatal, at 25° C. and was cultured at this temperature during the adult instars while producing the test mothers. Each of her eleven broods of offspring used in the test was divided into two temperature groups directly after release from the brood chamber, and these offspring were grown at the controlled temperatures until maturity. The temperatures used in this study were 25° and 14° C., the latter 2° higher than that used in the first series, since 12° C. was found to retard development greatly. A few of the test mothers were maintained at constant temperature throughout the tests. Others were transferred from one temperature to another directly after deposition of a brood of eggs in the brood chamber, in order that the embryos might be exposed to the new temperature of culture for the entire embryonic period. In contrast to the previous series in which the test mothers were transferred repeatedly from one temperature to the other, each test mother was transferred either just once or only after she had completed a number of instars at one temperature. This extended period of culture at one temperature was detrimental in one respect, in that the animal became so acclimated that successful transfers, particularly of embryos from low to high temperature, were almost impossible. Out of 80 broods transferred, only 15 survived. This difficulty was not encountered in the transfer of embryos from high to low temperature, probably since the low temperature appears to be nearer the optimum for this species.

In this series all offspring were graded directly at birth, reared to sexual maturity at room temperature, and then regraded. In spite of the difficulty of detecting the degree of male influence at birth in the secondary sex characters because of their minute size, this double grading showed clearly that the intersex phenotype is substantially fixed at birth.

The data for this second series are presented in Table 3 and show trends similar to those of the preliminary series: (1) There is a shift in grade of expression from 0.33, characteristic of culture at 25° C., to 0.73 for animals transferred from high to low temperature during embryonic development; (2) there is little or no shift in grade of expression in animals transferred from low to high temperature during embryonic development; (3) the grades for animals transferred from one temperature to another during ovarian development lie intermediate between those characterizing low- and high-temperature culture.

DISCUSSION

A time factor may account for the fact that a temperature change during embryonic development increased the intersex expression in *D. longispina* when transfer was made from high to low temperature but apparently had no effect when the transfer was made from low to high temperature during the same period. The embryos in the former case were almost instantaneously slowed in their development, so that the period of embryonic development lasted at least 4–5 days (even longer in the preliminary series, where the temperature was lower). In the reverse transfer, embryonic de-

velopment was accelerated to such an extent that it was completed within 48 hours. The temperature-exposure period is thus fully twice as long when the embryos are transferred to low temperature. This considerably longer period of exposure may be necessary for the alteration of processes involved in development of the intersex character.

During embryonic development, the organism is solely dependent on its own yolk supply for food, since eggs isolated from the mother soon after deposition maturation and yolk deposition in the ovary were transferred to a different culture temperature, still exhibited some effect of the previous culture temperature.

This carry-over of the effects of a previous temperature environment may persist even to the offspring produced a few instars after the transfer of the mother from the original modifying temperature. The most striking evidence for such a carry-over is found in the preliminary transfer series. The offspring undergoing complete development at 25° C.

TABLE 3

SECOND TRANSFER SERIES

Temperature Treatment	No. of Broods	No. of Offspring	No. of Offspring Described	No. of Males	Intersex expression				Average Grade of Offspring
					% Normal	% Slight	% Intermediate	% Extreme	
Complete development at 25° C..	377	3,400	2,302	0	67	32	1	0	0.33
Transfers from 25° to 14° C. during emb. dev.....	62	922	676	3	29	69	2	0	0.73
Transfers from 25° to 14° C. during ovarian dev.....	44	782	596	139	29	67	3	2	0.75
Complete development at 14° C..	142	1,891	1,478	2	18	75	5	2	0.91
Transfers from 14° to 25° C. during emb. dev.....	15	180	115	10	21	68	8	3	0.93
Transfers from 14° to 25° C. during ovarian dev.....	32	581	497	0	47	52	1	0	0.54

and washed free of any nutritive fluid present in the brood chamber were found to develop readily and normally outside the brood chamber in sterile pond water and in sterile synthetic pond water. This disposes of the postulate that alterations in the bacterial food supply of the mother due to temperature change is the determining factor in modifying intersex expression of offspring, at least during the embryonic period.

The data from the transfer experiments reveal an influence of temperature on the intersex character during the ovarian period, in that animals developing from eggs which at early stages of exhibited the high average grade of 0.58, as compared with the general average of 0.29 observed earlier as characterizing 25° C. culture. All these offspring, however, were produced by mothers which had been cultured for an extended period at low temperature. The offspring were produced one, two, or three instars after transfer of the mothers from low temperature and hence reflected some effect of early temperature history of mothers.

SUMMARY

1. It has been demonstrated that the temperature of the culture influences the expression of the intersex character in a genetically intersex clone of *D. longi-*

spina, Banta's line 1612. Animals reared at low temperature produce a larger proportion of intersex offspring which exhibit a fuller expression of male secondary sex characters than do their sibs cultured at higher temperatures.

2. Observations of cultures at 15°, 25°, and 29° C., and a few at 32° C., indicate that gradual suppression of the intersex character is obtained with increasing temperatures of culture, leading to complete inhibition of intersex expression at 32° C.

3. The study demonstrates that there is no short, well-defined temperature-effective period. If exposure to a modifying temperature is of sufficient duration, temperature may affect the expression during both ovarian and embryonic stages of development.

4. In the series of transfer experiments there is evidence for a carry-over of temperature effects extending to offspring produced several instars after transfer of the mother from the modifying temperature environment.

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THE EFFECTS OF CERTAIN DEPRESSING AGENTS ON INTRACELLULAR OXIDATION OF INDICATORS IN HYDRA

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IN THE course of a study of certain features of the axiate pattern of hydra by means of indicators (Child, 1947) it was found that the rate of oxidation of reduced dyes could be experimentally altered by depressing agents and that, below a certain limit of toxic effect, this alteration was completely reversible after return to water. The rate of oxidation of dyes was also found to differ with nutritive condition and was altered by a pathological condition in a hydra stock. The present paper brings together these data showing the sensitivity of indicator oxidation to differences in physiological condition.

MATERIAL AND METHODS

A California *Pelmatohydra*, perhaps *P. oligactis* (Child, 1947) was the chief material. *Chlorohydra viridissima* was also used to some extent. The experiments were terminated by the complete disappearance of hydra from the only local source known, and living material shipped from a biological supply house

was dead when received. In some cases the number of experiments is not so large as desired, but it is believed to be sufficient to demonstrate the effects of the agents used.

The chemical depressing agents to which the animals were subjected were KCN, LiCl, NaN_3, and NaF. In each experiment a single pair of animals from the same stock and as nearly as possible of the same size was used. One member of the pair was a control, remaining continuously in water, while the other was subjected for a certain time to a certain concentration of the agent, determined by preliminary experiment to be toxic but not rapidly lethal, except in some cases to tentacles and perhaps to stalk and foot and in a few animals to the hypostome. Different exposure periods and, except with NAF, different concentrations were used. The gradual decomposition of KCN in solution into HCN and KOH, with an increase of alkalinity and loss of HCN when open to air, makes certain precautions necessary in the use of this agent. Newly made KCN solutions were used, both without and with decrease of pH by means of dilute HCl. In some of the earlier experiments with KCN, 1 ml. of alkaline solution, containing a single animal, was left open to air during the exposure period. In later experiments, with a few exceptions noted below, the animal was brought at once into a glass cell containing approximately 0.5 ml. in the desired KCN concentration and covered, with complete exclusion of air. With the higher concentrations and shorter exposure periods, little or no difference in results was observed between the open and the covered solutions. With lower concentrations and exposure periods of 1 hour or more in 1 ml. of open solutions, slight and slow contraction and extension might reappear in animals which had been completely motionless earlier, indicating loss of HCN. KCN solutions with pH decreased by HCl show gradual increase in pH when left open to air. For example, 1 ml. of KCN M/400, with pH decreased to 6.8 and relatively large surface exposed to air, may show increase to pH 7.4 in 10–15 minutes. When covered, with complete exclusion of air, there is little or no appreciable increase of pH during the exposure periods used. In a few experiments with decreased pH, the solution containing the animal was left open to permit increase of pH. In connection with the question of a possible effect of KOH in KCN solutions, some data on effects of KOH and also of NaOH were obtained, and observations on intracellular pH were made by means of neutral red. With LiCl, NaN_3, and NaF no difference in effect was observed in solutions open to air and covered, with exclusion of air.

For dye oxidation, Janus green (diazine green) was found most satisfactory. Intracellular oxidation to red occurs in solutions completely reduced by sodium hydrosulphite. Intracellular oxidation of methylene blue does not usually occur or is very slight in *Pelmatohydra* in completely reduced solution. In *Chlorohydra* it occurs in light as a result of oxygen production by the green cells in the entoderm. After exposure to the agent, the experimental animal was washed in water and was at once brought with the control into a 0.5-ml. cell on a depression slide, containing Janus green completely reduced by sodium hydrosulphite, care being taken to avoid great excess of hydrosulphite. The cell was covered at once, with exclusion of air, and uniformity of oxygen content or lack was brought about and maintained by rapid circular to-and-fro movement of the slide about an axis vertical to its surface. This stirred the solution and in a few seconds again

reduced any of it which might have oxidized at the surface before it was covered. Also the position of the animals was frequently changed by gravity, since they rarely attach under experimental conditions. Since intracellular oxidation does not become evident for at least 2–3 minutes, except perhaps in the tentacles and since the animals were kept moving through the solution almost continuously during the first few minutes, regional differences in oxygen content in the solution certainly played little or no part in the results.

The concentrations of Janus green used were approximately 1/100,000 and 1/67,000. The pairs were observed almost continuously for 15–30 minutes except during change of position and "stirring" of the solution. When differences were slight, different illuminations and diaphragm openings and both transmitted and reflected light in partially darkened field were used.

For the indophenol reaction, solutions of the reagents were prepared as in earlier work (e.g., Child, 1944). The stock solution of para-aminodimethylaniline (dimethylparaphenylenediamine) was one small drop (approximately 40 drops = 1 ml.) in 10 ml. of water and 1 mg. or less of α-naphthol in 10 ml. of water. Some samples of aniline are solid but become liquid when slightly warmed. One small drop of each of these solutions in 1 ml. of water, or half of that concentration, was used with the animals. In one line of experiment the hydra was washed after exposure to KCN or the other agents and was then brought with the control into 1 ml. of water containing the proper concentration of the reagents. In other cases the reagents were added when the hydra had been brought into the depressing agent, in order to avoid possible recovery in water before intracellular indophenol became clearly evident, the intracellular reaction being slower than oxidation of Janus green. In these experiments the control and experimental animals were in equal volumes of water and the same concentrations of the indophenol reagents, but on different slides. Intracellular indophenol was found to be so highly toxic to hydra that cytolysis began early when the intracellular concentration, as indicated by color, was still low. Also the animals contracted progressively as the reaction proceeded, until in most cases body and stalk of *Pelmatohydra* formed a flattened disk with mouth widely open and entoderm more or less everted. With the extreme contraction, more or less reduction of indophenol might occur. Because of the difficulty in comparison of experimental and control animals which resulted from this contraction, the indophenol reaction has been less used than oxidation of Janus green.

Below a certain range of concentration and exposure period, the toxic effects of the four agents were completely reversible. Even after tentacles, stalk, foot, and hypostome were completely disintegrated, the post-tentaculate body might recover completely and undergo reconstitution. This paper includes data concerning the length of time required for recovery after return to water, as indicated by oxidation of Janus green compared with controls. These data were obtained by allowing the experimental animal to remain in water for various periods after exposure to the toxic agent and before it was brought, with the control, into reduced Janus green. Except for preliminary experiments to determine ranges of concentration of the toxic agents and exposure periods to be used for depressing effect, ranges permitting recovery, and some experiments with other dyes with essentially the same results, the data are

tabulated on the basis of comparison of the experimental member with the control of each pair.

POTASSIUM CYANIDE

GENERAL RESULTS

In all concentrations of KCN used, ranging from M/200 to M/4,000 with different exposure periods, the animals become relaxed and motionless, in the higher concentrations almost at once, in lower concentrations less rapidly. In the higher concentrations of alkaline KCN, cytolysis may begin at the tentacle tips in 5–10 minutes and progress basipetally. The tentacles may disintegrate completely, while other parts are still intact. In *Pelmatohydra*, stalk and foot are usually less susceptible than the tentacles but cytolyze earlier than the body. With decrease of pH, there is less or no cytolysis with a given concentration, even when exposure periods are much longer than with alkaline KCN; but, as will appear below, KCN near or at pH 7.0 is much more effective in decreasing the rate of oxidation of Janus green than the same concentration without decrease of pH. After the lower concentrations and shorter exposure periods, completely motionless animals, when returned to water with two or three changes, may show very slow and slight contraction within 3–5 minutes, but complete recovery requires a much longer time.

Tables 1 and 2 give data on the rate of oxidation of reduced Janus green and on the indophenol reaction for *Pelmatohydra* and *Chlorohydra* after KCN, as compared with controls. In each section of the tables the pairs are numbered consecutively in the order of decreasing concentration and increasing exposure period for each concentration. Table 1, Nos. 1–23, and Table 2, Nos. 1–15, give data for alkaline KCN without decrease of pH. In Nos. 24–29 of Table 1 and 16–25 of Table 2 the pH of the KCN solution was decreased as indicated. In No. 24 of Table 1 the pH was that of the water in which the hydra stocks were kept. In Table 1, No. 25, and Nos. 16–19 of Table 2 were left open to air during the exposure period, with gradual increase of pH. In Table 1, Nos. 30–36 and Nos. 26–29 of Table 2 give data for intracellular indophenol formation. In Nos. 33–36 of Table 1 and No. 29 of Table 2 the word "Reagents" following the concentration indicates that the indophenol reagents were added to both members of the pair at the time that the experimental animal was subjected to KCN. The column headed "Time in Minutes" gives the length of time in KCN. Since tentacles, body, and stalk with foot of *Pelmatohydra* and tentacles and body of *Chlorohydra* differ in susceptibility and in rate of oxidation, data for these regions are given in separate columns. Under "Tentacles" the word "Stumps" means that only basal stumps of the tentacles remain intact, the distal regions having undergone complete disintegration. Complete disintegration of tentacles and, in a few cases, of stalk and foot of *Pelmatohydra* is indicated by "0." The minus sign indicates that the region concerned oxidizes less rapidly in the experimental member of the pair after KCN than in the control; the equality sign means that there is no distinguishable difference between the two; and the plus sign means that the experimental member oxidizes more rapidly than the control. Repetition of the same sign in a column serves to indicate a distinctly greater difference than the single sign. Two different signs in the same column indicate that the rate of oxidation in the experimental animal as compared with the control differs in early stages of oxidation (the first 5–10 minutes) and later. For example, the

TABLE 1

Pelmatohydra: EFFECT OF KCN ON INTRACELLULAR OXIDATION OF JANUS GREEN COMPLETELY REDUCED BY SODIUM HYDROSULPHITE AND ON THE INDOPHENOL REACTION

(One Individual in KCN and One Control in Each Case)

Animal No.	Concentration	Time in Minutes	Tentacles	Body	Stalk
	KCN Followed by Reduced Janus Green				
1	M/200	5	+	+	+
2	M/400	15	o	−	=
3	M/400	15	Stumps−	−	−
4	M/400	15	Stumps−	−	−
5	M/400	15	Stumps−	−+	o
6	M/400	17	o	−	−
7	M/400	20	o	−	o
8	M/400	25	o	−	−
9	M/400	30	o	+	o
10	M/500	15	o	++	++
11	M/500	15	o	−−	=
12	M/500	15	o	−−	−−
13	M/500	15	o	−−	−−
14	M/500	50	Stumps−−	−−	−−
15	M/1,000	40	Stumps+	+	+
16	M/1,000	60	Stumps++	++	++
17	M/1,000	60	Stumps−	−	o
18	M/1,000	150	Stumps+−	+−	o
19	M/2,000	30	Stumps−	−	−
20	M/2,000	50	Stumps−	−	−
21	M/2,000	60	Stumps−−	−−	−−
22	M/4,000	75	+−	+−	+−
23	M/4,000	75	−	−	−
	KCN, Low pH, Followed by Reduced Janus Green				
24	M/400, pH 8.4–8.6	45	Stumps+	+=	−
25	M/400, pH 7.7–8.4	30	Stumps=	+=	−
26	M/200, pH 7.6	30	−	−	−
27	M/200, pH 7.3	120	Stumps−−−	−−−	−−−
28	M/200, pH 7.0	80	Stumps−−−	−−−	−−−
29	M/400, pH 6.6	30	−−−	−−−	−−−
	KCN and Indophenol				
30	M/400	10	o	−−	−−
31	M/400	15	Stumps=+	=+	+
32	M/400	30	Stumps−	−	=
33	M/500, reagents		−	−	−
34	M/500, reagents		−	−	−
35	M/500, reagents		−	−+	−+
36	M/500, reagents		−	−	−

body of the experimental member in No. 5 of Table 1 oxidized Janus green at first less rapidly, later more rapidly, than the control; in No. 18 the tentacles and in No. 22 the tentacles, body, and stalk oxidized at first more rapidly, later less rapidly, than in the control. The few cases of this sort perhaps indicate failure to attain uniform conditions rapidly enough in the solution; or some of them may conceivably result from differences in oxygen content of the cells or the digestive cavity in the two members. It must be emphasized that the data in the tables concern only the ectoderm. The entoderm of the tentacles and of the

TABLE 2

Chlorohydra: EFFECT OF KCN ON INTRACELLULAR OXIDATION OF JANUS GREEN, COMPLETELY REDUCED BY SODIUM HYDROSULPHITE, AND ON THE INDOPHENOL REACTION

(One Individual after Exposure to KCN and One Control in Each Case)

Animal No.	Concentration	Time in Minutes	Tentacles	Body
	KCN Followed by Reduced Janus Green			
1	M/400	7	Stumps−	+
2	M/400	15	Stumps+	+
3	M/400	15	o	+
4	M/400	15	o	+
5	M/500	15	−	+
6	M/500	25	+	+
7	M/500	45	o	−
8	M/800	10	+−	−
9	M/800	15	−	−
10	M/800	15	−	−
11	M/800	15 preceding oxidation in KCN	−=	−=
12	M/1,250	25	−	−
13	M/2,000	45 preceding oxidation in KCN	o	−
14	M/2,000	60	o	−
15	M/2,000	75	Stumps−	+
	KCN, Low pH, Followed by Reduced Janus Green			
16	M/400, pH 6.8–7.4	10	−	−
17	M/400, pH 6.8–7.4	20	+	−
18	M/400, pH 6.8–7.5	60	+	−
19	M/400, pH 6.6–7.4	60	−−	−−
20	M/200, pH 6.8	30	−	−
21	M/400, pH 6.8	30	−	=
22	M/400, pH 6.6	30	=	=
23	M/400, pH 6.6	30	−	−
24	M/400, pH 6.8	40	−	−
25	M/500, pH 6.8	180	−	−
	KCN and Indophenol			
26	M/600	12	o	=
27	M/800	10	−	−
28	M/800	15	o	−
29	M/800, reagents		−	−

stalk of *Pelmatohydra* oxidizes almost or quite as rapidly as ectoderm. The dark color of the body entoderm of *Pelmatohydra* renders comparison of the two members of a pair almost or quite impossible. In some cases indophenol formation in the body entoderm has seemed to be slightly less rapid after KCN than in the control in both species. With the low concentrations of Janus green used for intracellular oxidation, the green entoderm of *Chlorohydra* shows little or no color, except in the tentacles, where the green color is less deep. It seems possible that oxygen production in the body entoderm may be sufficient to oxidize Janus green completely to blue-green. In uninjured animals complete oxidation to blue-green has sometimes been observed in the body ectoderm of both species.

It is evident at once from Tables 1 and 2 that intracellular oxidation of reduced Janus green is by no means always less rapid after KCN than in controls. Even when tentacles are cytolyzed to stumps, it may be more rapid in the stumps and in other parts, as in Nos. 15 and 16 of Table 1 and No. 2 of Table 2. However, certain general results appear in the two tables. In Nos. 1–23 of Table 1 the minus sign appears 41 times, counting repeated signs as one; in 6 of these cases a plus sign also appears. The plus sign appears 18 times, in 6 with a minus sign. In other words, oxidation of Janus green is less rapid after alkaline KCN at all stages in approximately 64 per cent of the recorded cases and less rapid in either early or late stages in 11 per cent more. In the 9 cases of completely disintegrated tentacles it was probably less rapid while they were still alive. In the second section of Table 1, after KCN with decreased pH, oxidation is less rapid throughout than in controls in 78 per cent and very much less rapid in all cases with pH near or at neutrality. In the third section of Table 1, intracellular indophenol formation after, or in Nos. 33–36, with alkaline KCN is less rapid throughout than in the controls in 78 per cent.

Turning to the data on *Chlorohydra* in Table 2, the first section shows less rapid oxidation throughout after, or in Nos. 11 and 13 in, alkaline KCN than in controls in 52 per cent, less rapid oxidation in early or late stages in 9 per cent, and more rapid oxidation throughout in 36 per cent. These percentages seem to indicate that alkaline KCN is somewhat less effective in decreasing rate of dye oxidation than in *Pelmatohydra*. In this connection it is suggested that oxygen production by the entodermal zoöchlorellae may antagonize the KCN effect to some extent. It has been shown for other material that oxygen may decrease the effect of KCN.

In the second section of Table 2, after KCN with decreased pH, oxidation is less rapid throughout than in controls in 75 per cent, i.e., almost the same percentage as in Table 1, and more rapid in only 10 per cent, Nos. 17 and 18, both left open to air with increase of pH. As in Table 1, decrease of pH of the KCN solution increases the frequency of less rapid oxidation in the experimental members. The four pairs in the third section of Table 2 show less rapid oxidation after KCN in 83 per cent. Though the number of cases is not large enough to be significant, it is perhaps of some interest that the percentage of less rapid oxidation after KCN differs little from the 78 per cent with indophenol in Table 1. When the data for oxidation of Janus green and the indophenol reaction are combined, it appears that in both Table 1 and Table 2 these intracellular oxidations are less rapid than in controls during all or part of the observation period in approximately two-thirds of the intact

regions and the same as, or more rapid than, in controls in approximately one-third.

THE SIGNIFICANCE OF pH AND OTHER FACTORS IN RELATION TO KCN

The increase in effectiveness of KCN at pH levels near neutrality in retarding intracellular dye oxidation suggests that the KOH of alkaline KCN may play some part in decreasing the retarding action of cyanide on dye oxidation, either by increasing oxidase activity or in some other way. With pH at or near 7.0, most of the KCN has decomposed into HCN and KOH, and the KOH is neutralized by the HCl used to decrease pH. However, if the solution is left open, there is some increase of pH, evidently by further decomposition of KCN. Increase in amount of HCN with decrease of pH is probably a factor in the increased effectiveness of a given concentration of KCN at a pH near neutrality in retarding dye oxidation

It has been suggested that KCN may produce its effect on living organisms, not only by penetration of HCN after decomposition in the external solution but also, in part, by penetration of KCN in molecular form and decomposition into HCN and KOH within the cells. If this is the case, some increase in intracellular pH may result, unless the KOH is neutralized as rapidly as formed. In earlier experiments it was found that in KCN intracellular pH, as indicated by change in color of neutral red toward alkaline yellow, was slightly increased in *Paramecium*. This was regarded as indicating that KCN molecules entered the cell and decomposed into HCN and KOH inside (Child, 1945).

Pelmatohydra and *Chlorohydra*, brought into KCN M/500 or higher, with neutral red 1/40,000 or 1/50,000, do not differ in color from the controls during the first few minutes of staining in the same neutral red concentration, but the color in the ectoderm gradually changes slightly toward yellow. Animals brought into neutral red after a period in KCN usually show the change in color from the beginning of staining. Even with neutral red in neutralized KCN M/500 or M/200, in which most of the KCN is decomposed into HCN and KOH, intracellular neutral red may gradually become very slightly less red than in the controls. With KCN M/2,000 or M/4,000 no distinct difference in color of intracellular neutral red from controls has been observed. This suggests that, with these low concentrations of KCN, intracellular KOH is neutralized as rapidly as formed. The difference from the controls is not great in any case, and there are individual variations; but, with the higher KCN concentrations, it is sometimes sufficient to be visible to the naked eye in *Pelmatohydra*. With the approach or beginning of cytolysis, the color becomes completely yellow, evidently because the semipermeability is destroyed and KOH enters from the external solution. In KOH and NaOH solutions the animals stain red like controls, although the external solution is yellow. Evidently, KOH and NaOH do not penetrate at rates or in amounts sufficient to alter the color of intracellular neutral red. Since there is some change in color toward alkaline yellow in KCN solutions but not in KOH and NaOH, the conclusion seems to be justified that KCN penetrates in molecular form to some extent and decomposes within the cells. Probably more or less neutralization of the intracellular KOH occurs in all cases. In animals in water following exposure to KCN a secondary change in color of neutral red back to red seems often to be visible.

If intracellular KOH can in some way

decrease the effect of alkaline KCN in retarding oxidation of reduced Janus green to such an extent that, after relatively brief exposures to KCN, some individuals oxidize the dye more rapidly than controls and that, in general, oxidation is less retarded in alkaline than in neutralized or almost neutralized KCN, it might perhaps be expected that susceptibility would be less to alkaline than to neutral KCN. This, however, is not the case. Animals become relaxed, motionless, and insensitive to contact stimuli more rapidly and completely in alkaline than in neutral KCN of the same concentration. Moreover, a given concentration of alkaline KCN kills much more rapidly than the same concentration of neutral KCN, although the neutral solution is more effective in retarding dye oxidation.

In an attempt to throw light on the question whether the external KOH in an alkaline KCN solution has any effect on rate of dye oxidation, a few data were obtained concerning the effects on rate of oxidation of several concentrations of KOH and also of NaOH. The highest concentrations used were slightly below those which kill in a few minutes. These data appear in Table 3. After various periods of exposure to the bases, dye oxidation is more rapid in all regions in 67 per cent of the experimental animals, doubtfully more rapid in one animal. Counting the different regions separately, the percentage is the same. In only one case—3 per cent—(*Chlorohydra*, No. 4), and that after only 5 minutes in KOH, is dye oxidation less rapid than in the control. Examination of Table 3 indicates that the 33 per cent in which dye oxidation is doubtfully more rapid or less rapid than in controls or not

TABLE 3

EFFECT OF NAOH AND KOH ON INTRACELLULAR OXIDATION OF JANUS GREEN COMPLETELY REDUCED BY SODIUM HYDROSULPHITE
(One Individual after Exposure to NaOH or KOH and One Control in Each Case)

	Animal No.	Concentration	Time in Minutes	Tentacles	Body	Stalk
			Pelmatohydra			
NaOH.....	1	M/200	5	+	+	+
	2	M/400	20	+	+	+
	3	M/800	15	+	+	+
KOH.....	4	M/224	75	Stumps=	=	=
	5	M/280	30	+	+	+
	6	M/280	100	Stumps+	+	+
	7	M/1,008	100	+?	+?	+?
			Chlorohydra			
NaOH.....	1	M/200	30	+	+	
	2	M/400	30	=	+-	
KOH.....	3	M/280	15	Stumps+	+	
	4	M/375	5	=	-	
	5	M/560	15	Stumps+	+	

distinguishably different represent effects of extremes of concentrations or exposure times. It seems evident that these strong bases in external solution have some effect in increasing rate of dye oxidation, though they do not penetrate sufficiently to change appreciably the color of intracellular neutral red. The effect is apparently primarily a surface action. The bases may increase permeability, but reduced dyes penetrate so readily in any case that it may be questioned whether the effect of the bases on dye oxidation is primarily due merely to an increase in permeability. Until they are injured, the animals in KOH and NaOH appear more active than controls, as if stimulated or irritated. If intracellular KOH resulting from intracellular decomposition of KCN has an effect similar to that of KOH in external solution, it must increase, rather than decrease, susceptibility to KCN. Granting this, alkaline KCN should be more toxic than neutral, which is the case.

With decrease in the pH of the KCN solution, there is less or no KOH and less molecular KCN in the solution and consequently less intracellular KOH. According to Bodine (1924), HCN in acid, neutral, or slightly alkaline solution produces intracellular acidity because of its rapid penetration. No certain evidence of decrease in intracellular pH in hydra has been observed with neutral red, but slight intracellular color change of this dye toward the acid side is less distinct than the change with increase of pH. If intracellular pH is decreased in hydra by KCN at pH levels near neutrality, any resulting alteration in rate of dye oxidation will be in the opposite direction from that produced by increase in intracellular pH. Whether these suggestions are or are not correct, the second sections of Tables 1 and 2, as compared with the first sections, do show a higher frequency and a greater degree of retardation of dye oxidation; and comparisons of KCN concentrations and exposure times in the two sections, as well as other data not tabulated, also show that susceptibility to KCN with decreased pH is less than to the more alkaline solutions. Hydra sealed in KCN, with exclusion of air, live much longer in solutions at pH near neutrality than in the same concentrations and volumes at high pH.

LITHIUM CHLORIDE, SODIUM AZIDE, AND SODIUM FLUORIDE

Results obtained with LiCl, NaN_3, and NaF appear in Table 4. Only *Pelmatohydra* was used as material. Concentrations and exposure times, as determined by preliminary experiment, were within a range which permitted complete recovery of all parts except distal tentacle regions or entire tentacles (four cases in Table 4). Although numbers of animals are not large, it is evident at once from the table that dye oxidation after all three agents, and the indophenol reaction after LiCl and NaN_3, are less rapid than in controls, with few exceptions. The indophenol reaction was not used with NaF.

LITHIUM CHLORIDE

Lithium chloride, in the concentrations used, was less toxic than the other agents. Motility was decreased but not entirely inhibited, and the tentacles remained intact except in one animal. In three animals the tentacles oxidized Janus green after LiCl more rapidly than in controls; in the bodies and stalks of these animals and in all parts of other animals oxidation and the indophenol reaction were less rapid than in controls.

SODIUM AZIDE

Dye oxidation and the indophenol reaction were less rapid throughout after

sodium azide in 78 per cent of the data recorded in Table 4. In one animal (No. 2) oxidation was at first less rapid than in the control after a low azide concentration but later like the control. In No. 5 also after a low concentration, the body ectoderm was like the control. In Nos. 1, 3, and 4, dye oxidation and indophenol reaction are much less rapid after azide than in the controls. With the concentrations and exposure times used there was no cytolysis.

SODIUM FLUORIDE

Data are given in Table 4 only for NaF M/40; this concentration was found by preliminary experiment to be effective and to avoid most of the cytolysis occurring with higher concentrations and the doubtful differences with lower concentrations. According to the third section of Table 4, dye oxidation, even after 15 minutes in NaF M/40, as well as after longer periods, is always less rapid than in the controls. Basipetal

TABLE 4

Pelmatohydra: EFFECT OF LiCl, NAN_3, AND NaF ON INTRACELLULAR OXIDATION OF JANUS GREEN, COMPLETELY REDUCED BY SODIUM HYDROSULPHITE AND ON THE INDOPHENOL REACTION

(One Experimental and One Control Animal in Each Case)

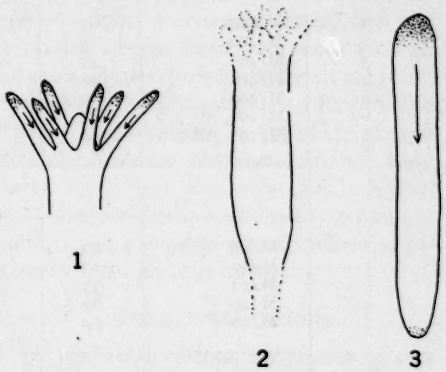
	Animal No.	Concentration	Time in Minutes	Tentacles	Body	Stalk
			LiCl			
Reduced Janus green.....	1	M/20	30	+	−	−
	2	M/20	30	−	−	−
	3	M/40	45	−	−	−
	4	M/40	60	+	−−	−−
	5	M/40	135	+	−	−
Indophenol...	6	M/20	55	Stumps−	−−	−−
	7	M/40	16	−	−	−
	8	M/40	45	−	−	−
			NaN_3			
Reduced Janus green.....	1	M/100	45	−−−	−−−	−−−
	2	M/500	120	−=	−=	−=
Indophenol...	3	M/25	50	−−	−−	−−
	4	M/167	30	−−−	−−−	−−−
		M/100	30			
	5	M/200	20	−	=	−
	6	M/200	60	−	−	−
			NaF			
Reduced Janus green.....	1	M/40	15	−	−	−
	2	M/40	15	−	−	−
	3	M/40	15	−	−	−
	4	M/40	25	Stumps−	−	−
	5	M/40	35	Stumps−	−	−
	6	M/40	60	o	−−	−−

tentacle cytolysis occurred with the longer exposure periods.

RECOVERY

It was found that the animals very soon became relaxed and almost or quite motionless in the concentrations of alkaline KCN, NaN_3, and NaF for which data are recorded in Tables 1, 2, and 4. Motility was less rapidly lost in KCN with pH near or at neutrality and was less completely inhibited in LiCl. After an hour or two in water following exposure to the lower concentrations of the agents, the animals were usually as active as, or more active than, the controls. After higher concentrations or longer exposure, recovery was less rapid; but, even after partial cytolysis and disintegration, intact parts could recover completely. After return to water, basipetal progress of tentacle disintegration ceased, the tips of intact basal tentacle stumps closed and after 24 hours in water showed an increase in rate of dye oxidation (Fig. 1). Even after complete disintegration of tentacles, foot, and stalk, and even the hypostome region in any of the agents used (Fig. 2), the rest of the body recovered in 20–24 hours, and its distal region showed a marked increase in rate of dye oxidation (Fig. 3). As will appear in another paper, the increase in rate of dye oxidation at the tips of tentacle stumps and distally in the intact part of the body after partial disintegration indicates initiation of reconstitution. Evidently, the toxic effects of the four agents are completely reversible in intact parts, even after complete disintegration of the more susceptible regions.

In view of these evidences of recovery, it seemed desirable to determine whether, or to what degree, recovery involved rate of dye oxidation. For this purpose, rate of dye oxidation in animals or in intact parts after various periods in water following exposure to one of the four agents was compared with rate in controls, as in Tables 1, 2, and 4. Data for *Pelmatohydra* are given in Table 5. A few experiments with *Chlorohydra* gave essentially similar results. In Table 5 the pairs of animals are numbered and arranged in order of length of time in water after various periods in the agent. This order, rather than length of time in the agent, has been used because it was known from the data of Tables 1 and 4 that all exposure times used were sufficient to produce toxic effects and, in most animals and regions, a decrease in rate of oxidation and because it seemed desirable to make evident any effect of length of recovery period in water. The plus, minus, and equality signs have the same meanings as in the other tables.



FIGS. 1–3.—Differential disintegration in the depressing agents and recovery after return to water. Fig. 1, recovery of tentacle stumps with initiation of reconstitution with greatly increased rapidity of dye oxidation at the tips, indicated by shading and basipetal progress of less rapid oxidation in more proximal regions, indicated by arrows. Fig. 2, intact part of body, indicated by unbroken outline; parts of disintegrated tentacles, hypostome and stalk, in which cellular debris attached to basement membrane still retains form in some degree, indicated by broken lines. Fig. 3, recovery of intact part of body of same individual as Fig. 2 after 24 hours in water, with initiation of reconstitution characterized by great increase in rapidity of dye oxidation at the distal end and slight increase at the proximal end, as indicated by shading.

TABLE 5

Pelmatohydra: RECOVERY IN WATER AFTER EXPOSURE TO KCN, LiCl, NaN_3, AND NaF AS INDICATED BY INTRACELLULAR OXIDATION OF COMPLETELY REDUCED JANUS GREEN IN EXPERIMENTAL ANIMALS AND CONTROLS

(One Experimental and One Control Animal in Each Case)

Animal No.	Concentration	Exposure Time in Minutes	Recovery Time in Hours	Tentacles	Body	Stalk
			KCN			
1	M/400	85	2/3	o	+−	+
2	M/400	35	2	Stumps+	+	+
3	M/400	45	2½	Stumps−	−	−
4	M/400	30	24	+	+	+
5	M/400	30	24	+	+	+
6	M/200, pH 7.0	85	½	+	+	+
7	M/200, pH 7.0	60	2¼	+	+	+
			LiCl			
1	M/50	90	1	=	=	=
2	M/40	15	2	+	+	+
3	M/40	95	2¼	+	+	+
4	M/40	60	4⅓	−=	−	−
5	M/20	90	21	Stumps+	++	+
			NaN_3			
1	M/100 / M/50	60 / 15	½	−	−	−
2	M/50	70	1⅓	−=	−=	−=
3	M/25	30	1½	=	+	o
4	M/50	30	2¼	−	−	−
5	M/25	90	19	Stumps+	+	+
6	M/25	40	20	Stumps+	+	+
			NaF			
1	M/40	60	½	−−	−−	−−
2	M/40	30	2	Stumps−=	=−	=−
3	M/40	30	2¼	Stumps=	=	=
4	M/40	75	2¼	=	=	−
5	M/40	15	2⅔	=	=	=
6	M/40	30	2⅔	=	=	=
7	M/40	30	2¾	o	−	−
8	M/40	30	20	Stumps+	=+	+
9	M/40	60	20	o	+	+
10	M/40	45	22	o	+	+

Comparison of Table 5 with Tables 1 and 4 shows a striking difference. It is evident at once that the plus sign appears much more frequently in Table 5 than in Tables 1 and 4. Although the percentage differences have no exact significance because numbers of animals, exposure times, and recovery times are different, the percentages for regions oxidizing Janus green as rapidly as, or more rapidly than, controls throughout the period of observation are given as having some interest. These are as follows: in the first two sections of Table 1, alkaline KCN and KCN with decreased pH, 22 per cent, in the first section of Table 5, 80 per cent; for LiCl in Table 4, 20 per cent, in Table 5, 80 per cent; for NaN_3 in Table 4, 0 per cent, or, if the indophenol data are included, since Janus green was used with only two animals, 5.5 per cent, in Table 5, 47 per cent; for NaF in Table 4, 0 per cent, in Table 5, 63 per cent.

Perhaps the most interesting point as regards recovery is that, with the progress of recovery, the rate of dye oxidation becomes not only equal to that in the control but higher. Recovery after KCN is rapid, the rate of dye oxidation becoming higher than in controls, with short recovery periods and after exposures which produce partial or complete tentacle disintegration (Table 5, Nos. 1, 2, and 6). After LiCl, No. 4 apparently recovered less rapidly than the others, but it might have been less rapid than the control originally. Recovery after NaN_3 is evidently less rapid, but after 19 and 20 hours in water (Nos. 5 and 6), even though the tentacles were partly disintegrated, all regions oxidize more rapidly than controls. The less rapid recovery is also evident after NaN_3 in the slower reappearance of motility. After NaF, rate of dye oxidation becomes like that in the controls in most cases after 2 hours or somewhat longer in water, but only after 20 hours does it become more rapid than in controls. In all cases in Table 5, rate of dye oxidation is higher than in controls after 19 hours or more in water (KCN, Nos. 4 and 5; LiCl, No. 5; NaN_3, Nos. 5 and 6; NaF, Nos. 8, 9, 10). The animals do not merely regain the original condition but go beyond it, much as if they had acquired an oxygen debt. Buchanan (1926) has observed a similar "overcompensation" in oxygen uptake during planarian recovery from KCN and has discussed its possible basis. The increase in rapidity of dye oxidation above the controls, not only after KCN but also after LiCl, NaN_3, and NaF, suggests an increase in oxidase activity as a general characteristic of recovery from all four agents.

OTHER OBSERVATIONS

A few observations on effects of certain other conditions on indicator oxidation are briefly noted. In *Pelmatohydra*, starved for 10–14 days and reduced to half or less of its original size, intracellular dye oxidation and the indophenol reaction are uniformly more rapid than in large, well-fed controls (5 pairs). On the other hand, animals distended by recently ingested daphnids oxidized more rapidly than controls containing no daphnids but not starved. In animals containing one or two recently ingested daphnids the ectoderm of the region containing a daphnid, and somewhat distended by it, oxidized Janus green more rapidly than adjoining regions. In earlier experiments on differential susceptibility to KCN it was found that ectoderm and entoderm of such regions disintegrated earlier than adjoining regions. Regions distended by exoskeletons of daphnids already digested were not increased in susceptibility (Child and Hyman, 1919). These results on dye oxidation and

susceptibility indicate that digestive activity alters the physiological condition of overlying ectoderm.

One stock of *Pelmatohydra* developed a pathological condition resulting in death. Small globular cell groups or rounded cells protruded from the ectodermal surface, progressing basipetally from the tentacle tips. The tentacles lost their reactivity to living crustacea and their contractility, decreased in length, and disappeared completely, and the animals finally died. From early stages of this condition on, intracellular oxidation of indicators was very much less rapid than in controls.

DISCUSSION

It appears from Tables 1–4 that the inhibiting or depressing action of the four agents, KCN, LiCl, NaN_3, and NaF, and the opposite effect of the strong bases, KOH and NaOH, can be made directly visible by means of intracellular oxidation of indicators. However, in the case of alkaline KCN, the high external or intracellular pH or both, perhaps also the potassium ion and, in *Chlorohydra*, oxygen production by the zoöchlorellae, may in some cases decrease or even overbalance the retarding action of KCN on intracellular dye oxidation. It seems not impossible that oxygen in the digestive cavity of *Pelmatohydra* may have some similar effect in some cases. However, since intracellular dye oxidation is almost uniformly less rapid than in controls after KCN with pH near neutrality, high pH appears to be the chief factor in increasing the rapidity of dye oxidation, that is, the high pH antagonizes the retardation of dye oxidation by KCN but increases the susceptibility of the animals to the agent. With the concentrations of alkaline KCN and the exposure t'mes used, the concentration of KOH is not high enough to account for the susceptibility to alkaline KCN. With the other agents the factor of pH is not involved, and retardation of dye oxidation and of the indophenol reaction (not used after NaF) occurs almost uniformly.

The rapidity of recovery of intact parts, even from toxic effects which produce partial or complete disintegration of tentacles and even of foot, stalk, and hypostome region, and the "overcompensation" in recovery are particularly interesting as indicating that the toxic effect is not merely completely reversible in intact regions but that the animal, in the course of recovery, attains a condition beyond or above the original.

While the work on hydra with indicators was in progress, a paper concerning the effects of respiratory inhibitors and stimulants on the organizing capacity of implanted hypostome pieces of *Pelmatohydra* appeared (Yao, 1945). That paper was responsible for a part of the observations and data on dye oxidation during recovery. Yao states that hypostome pieces subjected to KCN M/400 and M/200 for 30 minutes before implantation in other individuals were still capable of organizing action. Moreover, he found that hypostome pieces subjected to LiCl M/100 and M/50 for 30 minutes or to NaF M/40 for 15 minutes were also capable of organizing action after implantation. He concluded from these experiments that the metabolism of the hypostome is not concerned in its action as organizer. This conclusion is not justified because of the failure to consider the possibility of recovery. In view of the data of Table 5 and the observations on activity and initiation of reconstitution after return to water, it is evident not only that the toxic effects of the agents used by Yao and also the effect of NaN_3 are completely reversible but that within 24 hours, and in the case of KCN earlier,

there is not only complete recovery but an overcompensation as regards rapidity of dye oxidation. During this time, intact parts have regained full activity and show evidence of initiation of reconstitution. Incidentally, it may be noted that LiCl M/100 and M/50 are only slightly toxic to hydra with 30 minutes exposure. If metabolism of the hypostome, the high end of the body gradient (Child, 1947), is concerned in its organizing action, it may be even more effective after subjection to the agents for the periods used by Yao than without such treatment.

Yao speaks of keeping *Pelmatohydra* in KCN M/400 for times up to 24 hours. Animals in newly made KCN N/400, without exposure to air and consequent loss of HCN, die and disintegrate completely in 2–4 hours. It seems probable that Yao left his KCN solutions open to air or used old solutions which had lost HCN. He also maintains that implanted hypostome shows organizing action in water from which all oxygen has been removed by boiling, following by the use of sodium pyrogallate in a sealed container. The water was presumably exposed to air after boiling, and air must have been present in the container with the pyrogallate. The writer has used sodium pyrogallate in earlier experiments and has found that removal of all oxygen from water by this method is an extremely slow process. *Pelmatohydra* is highly sensitive to lack of oxygen. As noted elsewhere (Child, 1947), when all oxygen is removed by sodium hydrosulphite from a small volume of water containing an individual hydra and sealed, disintegration is complete in 2–4 hours at room temperature. That death is due to lack of oxygen, not to toxicity of hydrosulphite or its products, is readily shown by including a small air bubble in the sealed cell with the hydrosulphite and the hydra. In this case the animal lives for 2 or 3 days, according to size of the air bubble, even with a relatively high concentration of hydrosulphite. *Pelmatohydra* is certainly not anaerobic. Boiling and pyrogallate evidently did not remove all oxygen from the water rapidly enough to kill the animals within the period of Yao's experiment; consequently, it is not valid evidence for his conclusion that implanted hypostome can organize under anaerobic conditions.

One other line of experiment recorded in Yao's paper must be briefly mentioned. Proceeding on the basis that methylene blue increases respiratory metabolism, he implanted pieces from the subtentacular region after staining for 5–10 minutes in methylene blue 1/10,000. The assumption involved was that, if methylene blue does increase respiratory metabolism and if this is a factor in organizing action, the implanted pieces should become organizers. However, organization of a new axis occurred in only 3 out of 83 cases. With 5–10 minutes exposure to methylene blue 1/10,000, the hypostome does not become deeply stained in any *Pelmatohydra* material used by the writer; there is at present no evidence that this brief staining period increases metabolism appreciably or for any considerable length of time; consequently, the experiment remains, at best, inconclusive. Whether the three positive cases are significant is not evident. The writer has profound respect for anyone who can carry on scientific research in China under present conditions, and criticism of such research is an unpleasant task. However, it has seemed necessary in the present case.

SUMMARY

1. After subjection of *Pelmatohydra* and *Chlorohydra* to alkaline KCN in concentrations ranging from M/200 to

M/4,000 for periods of from 5 minutes to 2½ hours, according to concentration, intracellular oxidation of completely reduced Janus green and the indophenol reaction in the regions remaining intact are less rapid than in controls throughout or during a part of the observation period in approximately two-thirds of the intact regions and not visibly different from, or more rapid than, in controls in approximately one-third.

2. With decrease of pH of the KCN solution to a point near neutrality, frequency and degree of retardation of intracellular dye oxidation increase.

3. Slight intracellular change in color of neutral red toward yellow in KCN so'utions and absence of such change in intact animals in KOH suggest that some KCN enters the cells in molecular form and increases intracellular pH slightly by decomposition within the cells.

4. After subjection to KOH and NaOH, dye oxidation was more rapid than in controls in approximately 67 per cent, whether entire individuals or different regions were considered, although the strong bases did not penetrate rapidly enough to produce any distinguishable change in color of neutral red. With extremes of concentration or exposure time, dye oxidation was probably more rapid or less rapid than in controls or not distinguishably different in approximately 33 per cent.

5. The data indicate that, with alkaline KCN, increase in intracellular pH and perhaps also the high pH of the external solution are the chief factors in decreasing or overbalancing the retarding effect of cyanide on intracellular dye oxidation.

6. After exposure to LiCl M/20 and M/40 and to NaN_3 M/25–M/500 for various periods, dye oxidation and the indophenol reaction are less rapid than in controls in 80–100 per cent and after NaF M/40 in 100 per cent of the regions remaining intact.

7. Even after tentacles, stalk, and foot and, in some cases, the hypostome have disintegrated, toxic effects are completely reversib'e after return to water in the parts remaining intact. Recovery is indicated not only by reappearance of contradictility but by intracellular dye oxidation. With the concentrations used recovery after KCN and LiCl is more rapid than after NaN_3 and NaF. After all four agents, dye oxidation sooner or later becomes more rapid than in controls.

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RESPONSES OF FISH MELANOPHORES TO CHEMICAL WARFARE AGENTS[1]

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As part of an investigation of the effects of chemical warfare (C.W.) agents upon aquatic organisms, observations were made upon the reactions of melanophores of fish. These observations are of general biological interest, quite apart from their bearing upon the toxicity of C.W. agents and the biological detection of such agents.

MATERIALS AND METHODS

The materials used were sulphur mustard, three nitrogen mustards (β-chloroethyl amines), and lewisite. Sulphur mustard was introduced into aquaria in dioxane solution and lewisite in aqueous solution. The nitrogen mustards were introduced as the water-soluble solid hydrochlorides, which release free base in alkaline tap water (Gilman and Philips, 1946). No effects upon fish were noted in control solutions of dioxane at higher concentrations than those used with mustard.

Some of the observations on chromatophores were made upon fish in toxicity experiments, in which groups of ten 2-inch fish (bluegills or bullheads) were placed in 50-liter aquaria. Other experiments were designed specifically for chromatophore study, and the volume of solution varied according to the size of the fish. Chromatophores of dorsal and caudal fins were observed, either in fresh mounts or in permanent preparations after formalin fixation.

GROSS EXTERNAL EFFECTS

Fish which die in C.W. agents become covered with a coating of mucus, which does not appear to the same extent in controls killed by pithing. This slime secretion is particularly marked in lewisite (Figs. 1, 3, 4). Many other noxious agents also stimulate slime secretion.

Fish which die from natural causes develop opaque eyes after about a day in water, but fish in C.W. agents, particularly lewisite, show a milky cornea within a few hours after death. A large part of this milkiness is due to slime which covers the eye, but both the cornea and the lens also become opaque (Figs. 1–4). The opacity of the cornea and lens is not readily seen in small fish (2 inches or less in length). It was noted in large bullheads, bluegills, and crappies. When fish were killed by pithing and then put into lewisite higher in concentration than 1 p.p.m., they also developed opacity of cornea and lens.

PIGMENTARY REACTIONS TO C.W. AGENTS

The most obvious external reaction of fish to C.W. agents is color change. Chromatophores occur in the outermost

[1] The experiments described in this paper were conducted during 1942–43 under N.D.R.C. contract OEMsr-593, A. M. Buswell and Charles C. Price, technical representatives. This paper has been released for publication by the Public Relations Division, War Department Special Staff. F. B. Adamstone, consultant on the project, gave valuable advice.

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layers of the skin, even outside the scales and are, therefore, very exposed to vesicants. The following results are concerned largely with melanophores.

When a fish dies a natural death in nonpoisoned water, it gradually pales because of melanophore constriction so that, after a few hours or a day, it is thoroughly bleached in appearance. When a fish dies a violent death, as by pithing of the brain, there is an initial darkening due to melanophore expansion, probably initiated by discharge of the parasympathetic nervous system. After a few hours the usual paling reaction begins. Along with body paling, there is also a fading of eye pigments. These color changes in dead fish occur irrespective of background or illumination, although in field conditions the rate of fading may be affected by light and temperature. When putrefactive disintegration sets in, melanin may gradually disappear.

When fish die in water poisoned by C.W. agents, abnormal color reactions occur. The melanophores of living fish are also affected, but their responses are not so extreme as in dead fish because those concentrations which cause maximal melanophore reactions kill before the pigmentary reactions are complete. Death need not be due to poisoning by C.W. agents. In many of the following tests the fish were killed by pithing the brain before introduction into the poisoned water.

THE MUSTARDS

Those fish which died in water containing either sulphur mustard or nitrogen mustard or which were pithed and then placed in dilute mustard solutions darkened and remained dark until putrefaction was extensive. Figure 1 shows a photograph of a black crappie killed by sulphur mustard and left for 12 hours in the solution, compared with one killed by pithing and left for 12 hours in tap water; Figure 2 shows a crappie in solution of a nitrogen mustard, compared with one in tap water. The poisoned fish were dark, and showed more slime secretion than the controls. Sulphur mustard caused a grossly distinguishable dark condition in concentration of 5 p.p.m. or more. The threshold for scaleless fish (e.g., bullheads) was lower than for those with scales (e.g., bluegills). There was no effect of background or of illumination upon the response to C.W. agents.

Microscopic examination of the melanophores allowed detection at lower concentrations. In normal pale fish, either dead or alive, the melanophores appear as concentrated dark dots, which may be either irregular or smooth along their borders (Fig. 11). Normally expanded melanophores in a black bullhead are shown in Figure 5. Parker (1943) classified melanophores in order of increasing expansion as follows: punctate constricted, rough or intermediate constricted, intermediate, stellate expanded, and reticulate. Our fish showed another stage—fibrous expanded, as illustrated in Figure 6; the processes are very long, branched, and more attenuated than in the reticulate stage. Quantitative measures of melanophores were not practicable in the present experiments because changes in form were more important than changes in size; skin melanophores were not all affected to the same extent and at the same rate, and melanophores in different layers sometimes responded differently.

In sulphur mustard, the melanophores of fish killed by the poison or pithed and placed in solutions of it showed the following stages of response: (1) normal maximum stellate or reticulate expansion; (2) processes fibrous and often interlacing; (3) processes knobbed, especially at their ends; (4) pigment at ends

of processes, so that the centers of the melanophores often become empty and pale; (5) the knobbed processes broken, forming blobs of melanin; (6) breakage resulting in granulation. Finally, all identity of the original melanophores was lost, pigment granules might condense as flecks, and in decomposition the pigment was destroyed.

Whether a given fish showed all the above stages and the rate at which it passed through these stages depended upon the kind of fish and the concentration of mustard. Small (2-inch) black bullheads became totally granular in mustard within 24 hours. Bluegills were slightly less sensitive and often showed granulation and knobbed processes but not complete loss of melanophore identity. The melanophores might not be uniform over an entire fish. Areas of granulation and broken processes were diagnostic unless they were in the vicinity of skin abrasions. Basal portions of fins were more favorable for observation than fin tips. Using granulation and broken knobbed processes, concentrations of mustard at 1–2 p.p.m. could be detected with 2-inch bullheads. With bluegills of the same size the threshold for sulphur mustard was 2.5–5 p.p.m. The threshold was not sharp, but rather the degree of melanophore disruption increased with increasing concentration and time. For example, in black bullheads expansion due to sulphur mustard at 5–10 p.p.m. could be detected at 1–2 hours, and at 1–2 p.p.m. in 18–24 hours. A response could be detected within 6 hours in melanophores on the abdomen of pithed guppies in sulphur mustard at about 1 p.p.m.

Melanophore disruption was not observed in living fish, but the maximum fibrous expansion was seen, that is, the first two stages named above. Recovery frequently occurred after treatment in sublethal concentrations. Large fish (8 inches or longer) tended to recover more than did small fish; hence they appeared to have a higher threshold. In concentrations of mustard which required several days in the solution to kill (less than 10 p.p.m.), fish darkened while still alive and later recovered, as shown by constriction if on a light background. When they ultimately died, they paled. The mustard was effective only before it became hydrolyzed.

The chromatophores of the iris as well as those of the skin became expanded in mustard. In poisoned bluegills the yellow pigment cells of the skin, xanthophores, were well expanded. In nonpoisoned water, xanthophores of dead bluegills appeared as yellow flecks, while in water containing sulphur mustard the yellow was diffuse.

The nitrogen mustards had the same action qualitatively as sulphur mustard. The threshold for one of the more toxic of these in pithed bullheads was approximately 5 p.p.m. At this concentration expansion with knobbed processes and empty centers could be obtained within 6 hours, and granulation appeared later. In very high concentrations (over 100 p.p.m.) the fish were killed in less than 6 hours, and melanophores were normally expanded; the color cells appeared to be killed and fixed before they could respond.

LEWISITE

The action of lewisite upon pigment responses is more complex than that of the mustards, in that some fish and amphibia were made dark and other fish made pale by it. The species found to be darkened were as follows:

black bullhead, *Ameiurus m. melas*
yellow bullhead, *A. n. natalis*
channel catfish, *Ictalurus lacustris punctatus*
grass frog, *Rana pipiens*
tadpoles of *R. pipiens* and of bullfrog, *R. catesbiana*.

Those found to be pale in lewisite were as follows:

bluegill, *Lepomis m. macrochirus*
green sunfish, *L. cyanellus*
pumpkinseed, *L. gibbosus*
white crappie, *Pomoxis annularis*
black crappie, *P. nigro-maculatus*
largemouthed bass, *Huro salmoides*
warmouth bass, *Chaenobrythus gulosus*
common sucker, *Catostomus c. commersonnii*
guppie, *Lebistes reticulatus*

Figure 3 shows the effect of lewisite on a fish which was darkened, a channel catfish, and Figure 4 shows one which was made light, a black crappie, as compared with pithed fish in unpoisoned water.

The bluegills and other fish with melanophores constricted by lewisite could not be distinguished grossly from normal pale controls. In high concentrations these fish might even be lighter than controls (Fig. 4). On microscopic examination the bluegill melanophores were seen to be more constricted (Fig. 9) than in control dead fish. This lighter condition was seen in suckers at 25 p.p.m., in black crappies at 25 p.p.m., and in bluegills at 5 and 10 p.p.m. In guppies the melanophores constricted to an intermediate constricted condition within 2 hours and remained constricted; control dead guppies constructed more slowly and after 6–8 hours were usually punctate constricted. The threshold for intermediate constriction in guppies in lewisite was about 0.5 p.p.m.

In bullheads and tadpoles the manner of melanophore expansion was different in lewisite from that in mustards. In the bullhead there are two layers of melanophores, the smaller micromelanophores in the epidermis and the larger macromelanophores in the dermis. Parker (1934) has shown that these two layers respond similarly to changes in background. They also responded similarly to mustard, but they responded differently—at least at different rates—in lewisite. After a few hours in lewisite the macromelanophores were maximally expanded, while the micromelanophores were intermediate or constricted. Figure 10 shows expanded grayish macromelanophores and intermediate darker micromelanophores. The gross effect may be of splotchy expanded melanophores with blobs of melanin scattered through them. Later (10–20 hours) both layers expanded, and after 24–36 hours the melanophores were often broken up (Fig. 12). In lewisite the melanophore processes tended to be very broad and rounded at the ends and to have melanin extended, leaving empty or light centers. The threshold for this splotchy appearance in bullheads and tadpoles was about 0.1 p.p.m.

MANNER OF ACTION OF C.W. AGENTS ON MELANOPHORES

The preceding results show that the mustards cause maximum expansion of melanophores in a wide variety of fish and that lewisite causes expansion in some and constriction in others. That C.W. agents act upon living melanophores even in freshly killed fish is indicated by the following evidence: Typical reactions are seen in living fish, but they are less extensive than at lethal concentrations. Fish which have been dead for many hours before introduction into the C.W. agent, as well as injured areas, particularly on the fin tips of recently killed fish, usually fail to give typical responses to C.W. agents; hence the melanophores probably respond only if they are still alive. Responses were observed in fish in which the circulation had stopped and in fishtails cut from the body. Hence the C.W. agents need not be carried to the melanophores in the blood stream. That they can be carried in the blood, however, was shown by injecting lewisite intraperitoneally into several frogs; maximum darkening resulted. The threshold

for expansion of melanophores in living fish is, for some unknown reason, lower than that of the live melanophores in recently killed fish.

Parker (1934) has shown that when nerves going to an area of the skin of bony fish are cut, a dark band appears as a result of persistent nervous discharge arising at the point of injury. Such bands were produced by cutting one or two rays in the caudal fins of black bullheads, largemouthed bass, and green sunfish. When fish with such bands were pithed, the bands faded gradually. However, in living fish both sulphur mustard and lewisite obliterated the bands. Pithed bullheads showed melanophore granulation after about 15 hours in sulphur mustard (1 p.p.m. and higher) and splotchy, knobbed melanophores in lewisite (0.1 p.p.m. and higher), both in the band area and lateral to it. Pithed bass and sunfish showed constriction in lewisite in the band and at its sides. Thus it appears that C.W. agents, acting directly on melanophores, can overcome the action of nerve impulses. Black bullheads and largemouthed bass with tail bands were kept for 2 weeks to allow nerve degeneration. At the end of that time, sulphur mustard and lewisite elicited typical responses, both in the bands and outside them. It appears that C.W. agents act directly upon melanophores even though these melanophores lack circulation and innervation.

The fish which are made pale by lewisite have scales, while those forms (including frogs and tadpoles) which are darkened by lewisite lack scales. That the presence or absence of scales accounts for the difference in response to lewisite is unlikely, since the distal parts of the fins of most of the scaly fish lack scales and these areas may be affected like proximal regions. Those fish which are made pale by lewisite have their melanophores strictly under nervous control, if one may argue from the work of Parker and others on related species. In those forms which are darkened by lewisite the pituitary may cause melanophore expansion under certain conditions; in the frog, malanophore expansion is entirely hormonal (Parker, 1940; and other writers). However, the difference in manner of control can hardly account for the difference in response to lewisite, for this agent can act on noncirculated and on denervated melanophores. Injections of pituitrin were found to produce fibrous melanophores but not breakage or granulation in bullheads. It is concluded, therefore, that the melanophores of the scaly and the nonscaly fish, i.e., those constricted and those expanded by lewisite, must differ in their chemical receptor mechanisms. It is of interest in this connection that the micromelanophores of the catfish are constricted, while the macromelanophores are expanded at an early stage in lewisite action; later both are expanded.

SUMMARY

1. Sulphur mustard and three nitrogen mustards (1–5 p.p.m. and higher) caused maximal expansion of melanophores of bullheads, bluegills, crappies, guppies, and frogs. Melanophore processes became fibrous, knobbed, and finally broken.

2. Lewisite (0.1–0.5 p.p.m. and higher) caused maximal expansion of melanophores in two species of bullheads, channel catfish, frogs, and tadpoles; the same agent caused maximal constriction of melanophores in bluegills, two species of sunfish, black and white crappies, largemouthed and warmouth bass, suckers, and guppies.

3. Responses to C.W. agents were greater in fish or amphibians killed by

the agents or killed by pithing and then placed in solutions of the C.W. agents than they were in living individuals. However, the responses probably occurred only in living melanophores. Responses to C.W. agents occurred in melanophores which lacked circulation and innervation.

4. Lewisite permits the differentiation of melanophores into two effector groups in their response to direct chemical stimulation.

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PLATE I

FIGS. 1–4.—Photographs of fish killed by C.W. agents and left in solutions of those materials for approximately 12 hours, compared with fish killed by pithing and left for a similar time in tap water.

FIG. 1.—White crappies, control and fish killed by sulphur mustard at 100 p.p.m.

FIG. 2.—Black crappies, control and fish killed by nitrogen mustard at 100 p.p.m.

FIG. 3.—Channel catfish killed by lewisite at 25 p.p.m. and control.

FIG. 4.—Black crappie, control and fish killed by lewisite at 25 p.p.m.

FIGS. 5 and 6.—Photomicrographs of portions of the caudal fin of black bullheads. ×59.

FIG. 5.—Control, melanophores stellate expanded. ×59.

FIG. 6.—Control, melanophores constricted. ×59.

PLATE II

Photomicrographs of portions of the caudal fin. Magnification ×59.

FIG. 7.—Black bullhead in nitrogen mustard at 50 p.p.m.; melanophores fibrous and expanded.

Fig. 8.—Black bullhead in sulphur mustard at 25 p.p.m.; melanophores granular and broken.

FIG. 9.—Bluegill in lewisite at 5 p.p.m.; melanophores constricted.

FIG. 10.—Black bullhead in lewisite at 10 p.p.m.; macromelanophores (*macro*) splotchy, expanded; micromelanophores (*micro*) constricted.

FIG. 11.—Yellow bullhead control; melanophores constricted.

FIG. 12.—Yellow bullhead in lewisite at 5 p.p.m.; melanophores splotchy and expanded.

PLATE I

FIG. 1

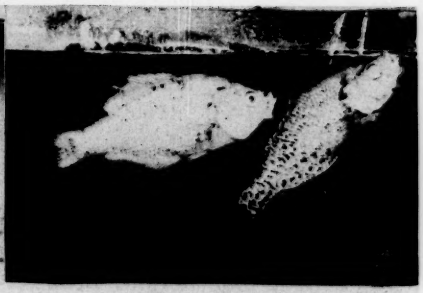

FIG. 2

FIG. 3

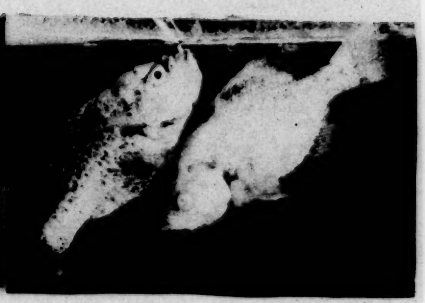

FIG. 4

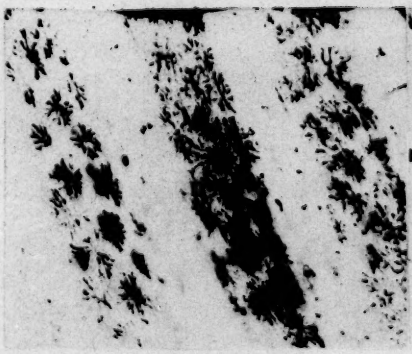

FIG. 5

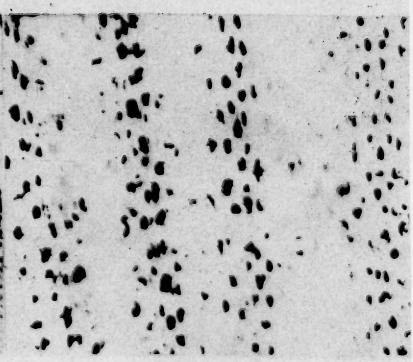

FIG. 6

PLATE II

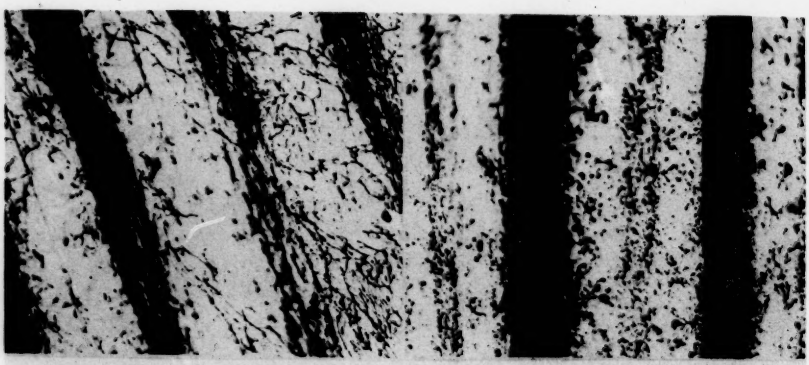

FIG. 7 FIG. 8

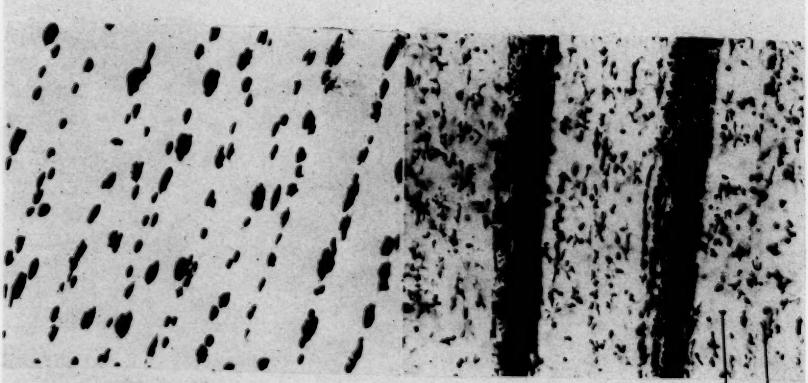

FIG. 9 FIG. 10

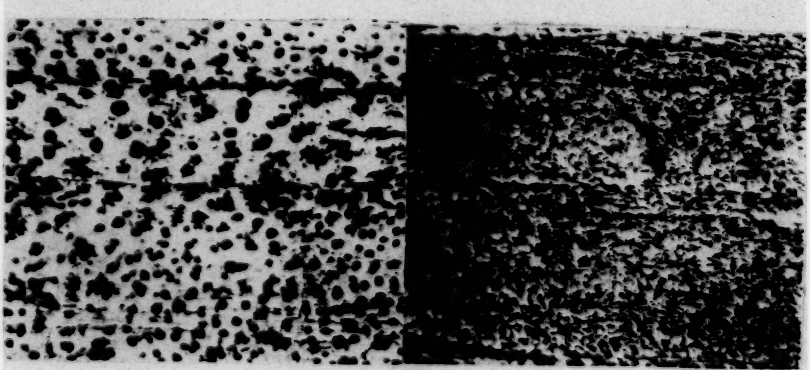

FIG. 11 FIG. 12

EFFECTS OF TESTOSTERONE PROPIONATE ON SEX DEVELOPMENT IN FEMALE AMBYSTOMA LARVAE[1]

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INTRODUCTION

It has been observed that anuran larvae differentiate as males if treated with androgens and as females when estrogens are administered, regardless of their genetic sex. If older, well-differentiated larvae are similarly treated, the females may still be reversed to males, but males do not respond to estrogens by a transformation to the female sex. Presumably this is due in part to an inadequacy of the cortical element remaining in the sex gland of the male (literature reviewed by Mintz, Foote, and Witschi, 1945).

The response of urodeles to sex hormones presents a somewhat different picture. In 1938, Burns reported that estrone injections in *Ambystoma maculatum* (syn. *Amblystoma punctatum*) larvae cause genetic males to develop gonads, which, by the time of completion of metamorphosis, resemble juvenile ovaries. Similarly, Ackart and Leavy (1939) obtained reversal of *A. tigrinum* larvae with estrogens, and Hanaoka (1939) observed occasional medullary germ cells differentiating in the female direction in *Hynobius retardatus* males injected with follicular hormone. Burns further concluded, in 1939, that testosterone propionate injections into *A. maculatum* larvae over a period of 45 days cause varying degrees of intersexuality in developing ovaries. He regards 4 of his 11 experimental larvae as genetic males, the remaining 7 as genetic females transforming into males. All these studies were initiated at stages after sex differentiation had begun. Foote, in 1940 and 1941, performed a number of experiments with *A. maculatum*, *A. tigrinum*, and *A. opacum*, in which emphasis was placed on early hormonal treatments, starting shortly after hatching and before the inception of sex differentiation. The hormone was added to the aquarium water rather than being directly injected. He confirmed the finding that estrogens cause genetic males to develop into hermaphrodites or females. He is, however, led to the conclusion that testosterone propionate likewise induces a definite shift of the sex ratio in the female direction (70 females, 45 hermaphrodites, 14 males); therefore a feminizing action of the hormone is postulated. Galgano (1942) reported slow degeneration but not inversion of the ovaries of testosterone propionate–treated *Triton cristatus* adults.

As this review indicates, the effect of androgenic hormones on sex differentiation in salamanders is still a controversial subject. Since neither Burns nor Foote knew the sex of individual animals at the start of their experiments, the genetic sex of the treated animals remains somewhat in doubt. To clarify this situation it

[1] Aided by grants from the National Research Council, Committee for Research in Problems of Sex; grant administered by Professor Emil Witschi. This investigation was undertaken at Professor Witschi's suggestion and carried through with his helpful advice and criticism. A preliminary report has appeared (Mintz, 1946).

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seemed desirable to conduct a series of experiments with animals of known genetic sex.

MATERIALS AND METHODS

Ambystoma mexicanum and *A. mexicanum-tigrinum* hybrid larvae of known pedigree which were exclusively genetic females were used.[3] One of the lots obtained consisted entirely of ZW females; others included 50 per cent ZW and 50 per cent WW females. For a description of the manner in which these females were procured and their chromosomal constitution determined, the reader is referred to the publications of Humphrey (1942 and 1945). There were white, as well as darkly pigmented, individuals among the animals used.

The experiments fall into two general groups: the first deals with testosterone propionate[4] treatment of young females starting at various times shortly after hatching and before visible sex differentiation; the second involves administering the same hormone to older larvae (5 months of age) which already possess well-differentiated ovaries. For convenience, these are designated "Group A" and "Group B," respectively. The older larvae were easily and more economically treated by the injection method, whereas, owing to the small size of those in Group A, the method of adding the hormone to the aquarium water was given preference.

Group A consists of three series which differ in size, genetic constitution, and age of the animals at the time treatment was initiated (Table 1).

The larvae were kept singly in 100 ml. of previously aerated tap water. Each series was divided into four sets of animals. The first set served as untreated controls; the other three received 0.05, 0.01, and 0.002 mg. of hormone per 100 ml. of aquarium water per day (0.5, 0.1, and 0.02 mg. per liter), respectively. Crystalline testosterone propionate was made up in solutions of 95 per cent ethyl alcohol, and 5 drops were added daily from calibrated hypodermic syringes to bring the administered amount to required concentrations. The 100-ml. level was maintained by daily additions of water to replace loss through evaporation, and the entire contents of each dish was changed every third day, since repeated administration of hormone probably produces a change in the hormone concentration. Foote (1941) concluded from rat bioassays of estrone-containing aquarium water that the amount of hormone actually taken from the water by the larvae is very small and that daily addition of equal quantities of this hor-

TABLE 1

SERIES OF GROUP A

Series	Age, in Days after Hatching	Number of Larvae	Total Length at Start of Experiment	Sex Chromosomal Constitution
I	63	72	18–20 mm.	50 per cent ZW, 50 per cent WW
II	54	118	17–20 mm.	100 per cent ZW
III	15	105	14–16 mm.	50 per cent ZW, 50 per cent WW

[3] The author wishes to express her appreciation to Professor R. R. Humphrey, of the University of Buffalo, who has been of the utmost assistance in supplying these larvae.

[4] Thanks are due the Ciba Pharmaceutical Products, Inc., for their generous supply of testosterone propionate (Perandren).

mone results in almost trebling the concentration at the end of each 3-day period. No similar tests have been conducted on the rate of disappearance of testosterone.

Treatments were begun in March and continued over a period of approximately 10 weeks. Twelve animals were then maintained for another 5½ months without further treatment. Their subsequent history is outlined in Table 2.

Group B consists of 166 larvae, 50 per cent of ZW and 50 per cent of WW chromosomal constitution. When treatments were initiated, they were 5 months old and 40–100 mm. (most being 70–80 mm.) long. Five of these were preserved as controls at the start of the experiment. Testosterone propionate was administered by intraperitoneal injections of sesame oil solutions (0.05 ml. sesame oil per injection). The animals were kept separately in approximately 500 ml. of water, changed once a week. Of 161 animals, three groups of 40 each received 0.25, 0.05, and 0.01 mg. of hormone per injection. Of the 41 controls, 20 were given injections of 0.05 ml. sesame oil per week. The injections were begun in May and made once a week, alternately on the animals' right and left sides. After 10 weeks, 114 larvae then remaining were preserved in Bouin's fixative (47 had died and were preserved during the 10-week period).

The temperature of the amphibian room usually remained close to 20° C., but extremes of 15° and 22° C. were recorded during the course of the experiments.

RESULTS

GENERAL DEVELOPMENT

There is no significant size difference in any of the experimental sets as compared with their controls.

TABLE 2

GROUP A LARVAE MAINTAINED BEYOND INITIAL 2½ MONTHS OF EXPERIMENT

Number of Animals	First 2½ Months*	Treatment at 8 Months of Experiment (Age 8½–10 Months), Following 5½-Month Lapse	Treatment at 9 Months of Experiment	Treatment at 9½ Months of Experiment
1	C	Preserved, 70–80 mm. length	.....	.....
2	T			
3	C	C†	.....	1 given 0.3 ml. alcohol/liter/day‡
2	C	T (0.5 mg/liter/day)†	.....	Raised to 1 mg/liter/day
4	T	T (0.5 mg/liter/day)†	1 raised to 1 mg/liter/day	Same animal raised to 2 mg/liter/day; remaining 3 raised to 1 mg/liter/day

* C, untreated controls; T, experimentals which received hormone during the first 2½ months of the experiment.

† These 9 larvae were preserved at varying intervals up to as late as the 16th month of the experiment (age 16½–18 months).

‡ This was the maximum amount of alcohol in which hormone given to treated animals was dissolved.

Mortality was especially high in Group A, but was about the same in control and experimental series. Animals which died were quickly preserved and, as a rule, were still usable. Moreover, mortality rates fortunately do not affect experimental findings in these all-female groups.

A striking feature observed among a large proportion of experimentals in both Groups A and B, but not among their controls, was the development of a pronounced ascites—tremendous distention of the body cavity due to accumulation of fluid (Fig. 3). After one-third of the 10-week experimental period of

Group B had elapsed, all untreated, as well as all oil-injected, controls were normal, while approximately 10 per cent of each series of hormone-injected larvae were ascitic. These figures increased as the experiment progressed. Such animals were punctured from time to time to allow the fluid to escape.

No differences were observed in alcohol-treated or oil-injected controls as compared with corresponding untreated controls.

GONADS

The gonads of all experimental animals remain ovarian in character, both grossly and microscopically. No intersexes or hermaphrodites are found.

Group A.—Normal ovarian differentiation and development is not interfered with in animals of Group A, receiving male sex hormone before and during the period of sex differentiation, except for a slight growth lag as compared with controls (see Figs. 4 and 5).

Group B.—In Group B, one oil-injected control has a small right ovary, the middle third of which is reduced to a thin longitudinal strip containing only a few germ cells. No other deviations occur among controls. In testosterone-injected larvae, however, 15.8 per cent have ovaries whose cortical component is markedly reduced. One individual receiving 0.01 mg., 5 at 0.05 mg., and 13 at 0.25 mg. per week have ovaries containing only a few gonia and ovocytes scattered in the cortex, and greatly enlarged ovarial sac cavities. These sex glands have become thin-walled, fluid-filled sacs, with germ cells sparsely distributed in the wall (cf. Figs. 9 and 10). Further ovocyte formation from ovogonia is inhibited, but no destruction of synaptic ovocytes and auxocytes occurs as was reported for testosterone-treated anuran females (Mintz, Foote, and Witschi, 1945). Synaptic ovocytes present at the start of the experiment are evidently not hindered in becoming auxocytes. It is impossible to tell whether the animals which show this response belong consistently to one or the other of the genetic types or are evenly distributed between the two. Altogether, it may be said that none of the observed results indicates a qualitative or quantitative dissimilarity in the reactions of ZW and WW females to the hormone.

MESONEPHRIC KIDNEYS

Group A.—Although no animals treated during early larval life show any ovarian abnormalities, a number have undergone changes in mesonephric structure. By the end of the $2\frac{1}{2}$ months of preliminary treatment with testosterone propionate, many of the experimentals reveal a slight enlargement of the mesonephric tubules (hydronephrosis), presence of cystic tubules, and a reduction in the number of tubules and blastema cells (Fig. 5). These changes occur with equal frequency among the three series, as well as among the three dosage sets in each series.

The most severe kidney damage appears in 6 larvae of Series I which had received 0.5 or 0.1 mg. of hormone per liter. These animals had remained alive during the $5\frac{1}{2}$-month period when treatment was discontinued. By this time they had grown considerably in size (from 30 to 70–80 mm. total length), and much of the kidney damage is easily visible in gross dissections. The deleterious effect of the hormone upon the mesonephros is irreversible and, once established, becomes relatively more exaggerated as the animal increases in size. Two of the 6 animals were killed before treatment was resumed (see Table 2); both lack kidney tissue for a length of 2.5 mm.

below the gonads, with cystic mesonephroi anterior and posterior to this level. The sex ducts continue through this region, however (Fig. 7). A cross-section through the left kidney of the same larva as in Figure 7, taken below the region where the mesonephros is completely deleted, shows the enormous distention of tubules and the spread of ciliated epithelium over the peritoneal surface (Fig. 8). The other four animals had again received testosterone propionate after the $5\frac{1}{2}$-month interval (Table 2); they were killed at various dates thereafter. All show extensive damage and gaps in the lumbar kidneys for stretches of 2–3 mm., with kidney depletion in remaining regions. The animal which received the highest dose (2 mg. per liter per day) exhibits the most spectacular reduction of all; in this individual, both mesonephric bodies are almost completely destroyed, with only a few discrete clumps of cystic tubules remaining on each side. Some of the tubules present are degenerating, and one is filled with blood. The control to which 0.3 ml. of alcohol per liter per day was administered has normal kidneys, indicating that the effect is due to the hormone and not to the alcohol solvent.

Group B.—Older larvae receiving testosterone propionate show similar, though less extensive, kidney changes (Fig. 11). The occurrence of cortical reduction and ovarial sac enlargement in the sex gland is paralleled by reduction of mesonephric tubules and blastema, as well as occasional dilation of tubules. None of these larvae shows the discontinuous kidney condition observed in Group A, possibly because the experiment was started later and carried on for a shorter period of time. Kidney reduction also occurs in some animals not having the loss of cortex reported in the ovaries.

PRONEPHRIC KIDNEYS AND PRONEPHRIC DUCTS

Pronephric bodies and ducts normally disappear in *Ambystoma* during larval life, but their regression is partly prevented by treatment with testosterone propionate.

The pronephric system was present in animals of both groups when the experiment was begun. Normal involution occurs between the ages of 8 and 10 months (70–80 mm. total length) in Group A controls and between the ages of 5 and $7\frac{1}{2}$ months (70–80 mm. total length) in controls of Group B (Fig. 12). Inasmuch as no hormone was administered during the period of pronephric regression in Group A, the conditions in this group afford no opportunity for studying the effects of androgen on disappearance of the kidneys and ducts. In Group B, however, the time of normal pronephric loss coincides with the 10-week period of treatment in experimentals.

Group A.—The pronephric ducts do not respond to this early hormone treatment.

Group B.—Most of the treated animals still retain pronephric bodies and all or parts of the ducts at the end of the experiment. Some have only one pronephric duct; others have discontinuous segments of one or both ducts (Fig. 1, *d*, *g*, *h*, *i*). Remaining intact portions of the ducts are slightly stimulated, as seen by thickening of the wall and enlargement of the lumen (Fig. 13).

The camera lucida drawings in Figure 2 reveal an interesting situation regarding the question of stimulation of the pronephric ducts by testosterone propionate. The condition observed in Figure 2, *a*, is typical of that found in many treated animals of Group B at the end of the experiment, i.e., a part of the pronephric ducts is retained and only slightly stimulated, whereas the oviducts

are very greatly hypertrophied by the hormone. Figure 2, *b*, illustrates a case in which the character of the ducts on the animal's right side is similar to that in Figure 2, *a*, while on the animal's left no part of the oviduct has formed (this is the only instance of complete oviduct absence) and the pronephric duct is highly stimulated and covered with vesicular excrescences. Figure 2, *c*, is taken from Witschi (1942) and shows the anterior gonoducts of a testosterone propionate-injected tiger salamander in which downgrowth of both oviducts has been arrested near the ostial region by the hormone. The retained pronephric ducts are bilaterally stimulated to the same extent as the left pronephric duct in Figure 2, *b*.

MESONEPHRIC DUCTS

Group A.—Mesonephric duct stimulation is slight in the younger animals during the first period of treatment, and only the most caudal parts of these ducts frequently exhibit a distinct enlargement. In two larvae, small budlike structures resembling seminal vesicles are present at the lower regions of the stimulated mesonephric ducts (Fig. 6). Maintenance of a hypertrophied condi-

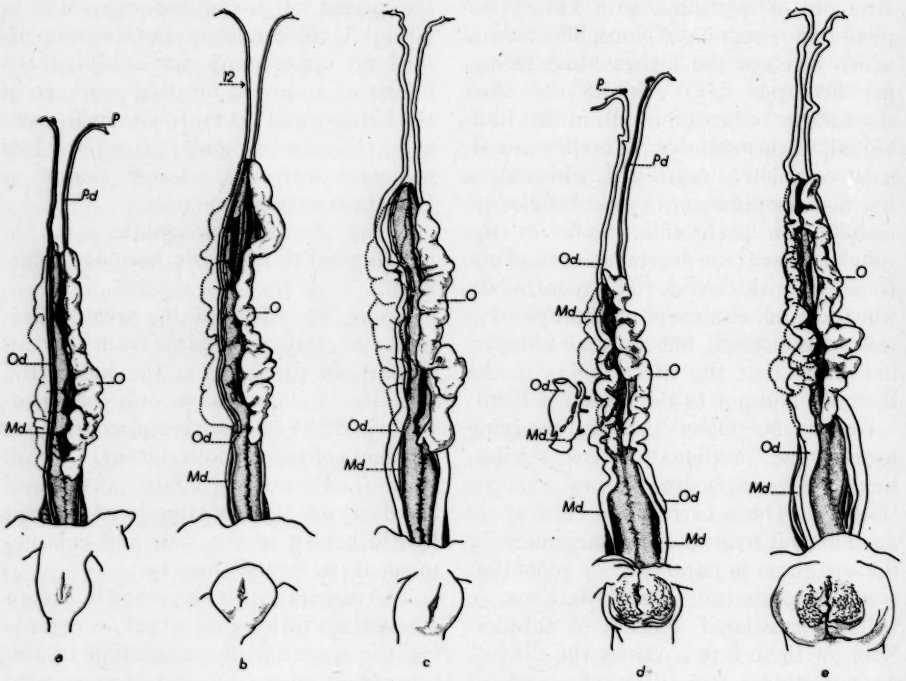

FIG. 1.—Urogenital organs (ventral views) of control and testosterone propionate–treated female larvae. Animal *a* preserved 3 weeks after start of the experiment; the remaining animals preserved after 10 weeks, at termination of the experiment. *a*, *b*, *c*, uninjected controls; *d*, *e*, *f*, injected with 0.01 mg. of hormone per week; *g*, injected with 0.05 mg. of hormone per week; *h*, *i*, injected with 0.25 mg. of hormone per week. *O*, ovary; *Od*, oviduct; *P*, pronephros; *Pd*, pronephric duct; *Md*, mesonephric duct. Outline of right ovary dotted in, with underlying structures showing through; mesonephros stippled; fat bodies in black. Camera lucida drawings, ×3. Semidiagrammatic enlargements of fusion places where oviduct enters mesonephric duct. Arrows indicate cross-sections with figure number noted.

tion of the mesonephric ducts apparently depends upon continuance of hormone treatment. Only 1 animal (which had previously received 0.5 mg. of hormone per liter, the highest dose) still showed slightly hypertrophied ducts by the end of the $5\frac{1}{2}$-month period during which no hormone was given. Evidently, the mesonephric ducts are more competent to respond to the hormone at a later larval stage, for later treatments produce a high degree of stimulation within 3 months and possibly in a shorter time.

Group B.—Among the injected group, the extent of stimulation of the mesonephric ducts depends on the dose which the animal receives. Untreated and oil-injected controls have thin, and occasionally slightly wavy, ducts at the end of the experiment. All hormone-injected individuals have grossly enlarged ducts, with thickened walls and enlarged lumens. The number of convolutions increases with dosage. This stimulation progresses if hormone administration is continued. After five injections the ducts are scarcely different from those of controls and assume the hypertrophied character only with prolonged treatments.

The mesonephric ducts are more responsive to hormone administration than are the pronephric ducts. Further aspects of mesonephric duct change will be discussed below in connection with the oviducts.

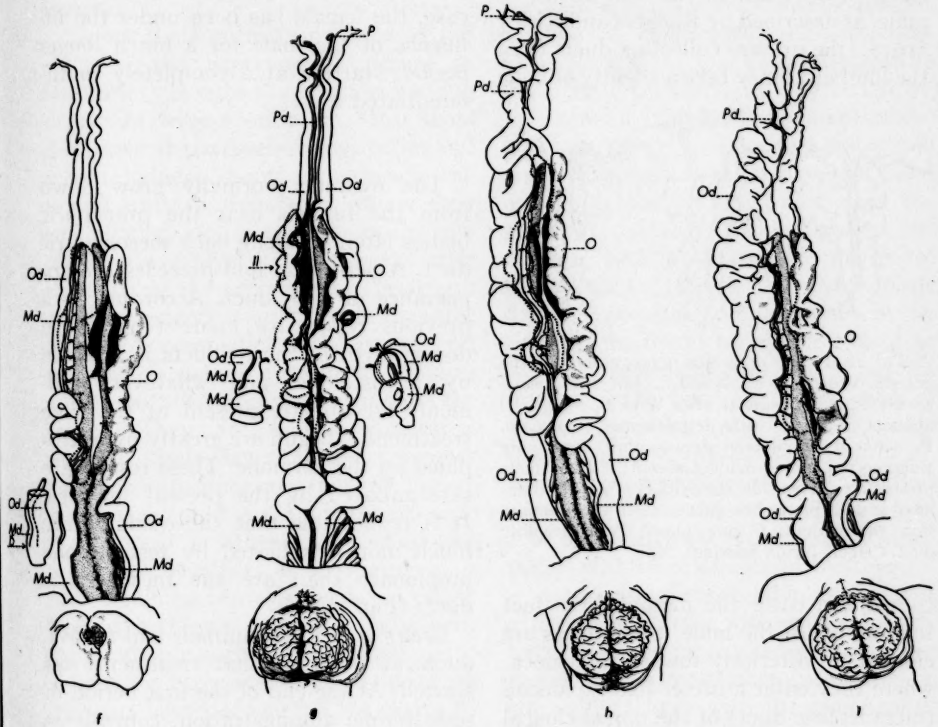

FIG. 1.—*Continued*

URINARY COLLECTING DUCTS

In control females, the short urinary collecting ducts of the sexual kidney enter the mesonephric ducts at regular intervals, and this arrangement is maintained in experimental females.

There is a sexual dimorphism in the pattern of the posterior urinary collecting ducts in urodeles. In the *Ambystoma* female, as described by Rodgers and Risley (1938), the urinary collecting ducts from the lumbar kidney take a slightly oblique course and enter the mesonephric duct separately. In the male, these ducts are elongated posteriorly toward the cloaca, where they enter a ureter-like expansion (metanephric duct) of the dorsal cloacal wall; this, in turn, opens into the mesonephric duct at the end of a urogenital papilla on each side.

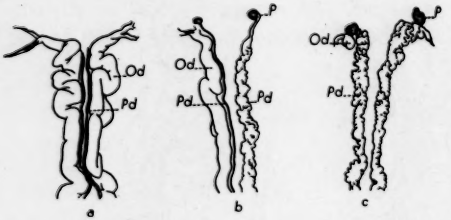

FIG. 2.—Female urogenital organs anterior to the gonads. Ventral views. *a* and *b*, *Ambystoma mexicanum*; *c*, *A. tigrinum* (*c* after Witschi, 1942). All animals injected with testosterone propionate. Pronounced stimulation of pronephric ducts only when corresponding oviduct absent; if both ducts present on a given side, the oviduct is highly stimulated and the pronephric duct receives little stimulation. *Od*, oviduct; *P*, pronephros; *Pd*, pronephric duct. Camera lucida drawings, ×3.

Group B.—All controls exhibit the typical female pattern. Testosterone-injected animals possess enlarged collecting ducts which show posterior migration and approximate the male pattern by opening singly or in groups near the caudal end of the mesonephric duct. The male pattern is simulated but never fully achieved, i.e., the migration does not progress as far back as the cloacal wall, nor do all the collecting ducts unite with a metanephric duct where they anastomose with the mesonephric duct. Witschi (1937) describes the occurrence of the typical male collecting duct arrangement in a sterile *jeffersonianum* salamander female united in parabiosis with a mature *tigrinum* male. In this case, the female has been under the influence of the male for a much longer period, starting at a completely undifferentiated stage.

OVIDUCTS

The oviducts normally grow down from the funnels near the pronephric bodies laterally along each mesonephric duct. A peritoneal fold precedes the appearance of each duct. According to a previous report by Foote (1941), the downgrowth of the oviducts is arrested by testosterone, and whatever rudiments are already present at the time treatment is begun are greatly hypertrophied by the hormone. These results are substantiated by the present findings. It is remarkable that the oviducts are much more stimulated by testosterone propionate than are the mesonephric ducts (Fig. 14).

Group A.—These animals had no oviducts at the time that treatment was started. At the end of the first period of testosterone administration, controls as well as experimentals still lacked oviducts. The interval during which hormone was not given (between $4\frac{1}{2}$ and 10 months of age in Series I, and 3–$8\frac{1}{2}$ months of age in Series III) apparently coincides with the normal period of growth of the oviducts in Group A, for these ducts now appear in control and in previously treated animals, being of similar unstimulated character. It is therefore impossible to form conclusions in this particular group regarding the in-

hibitory influence of the hormone upon oviduct downgrowth. Resumption of treatment soon produces enormous hypertrophy of the oviducts in experimental subjects, with tremendous thickening and folding of the wall (accounted for chiefly by enlargement and increase in the number of mucous or mucin-secreting glands) and enlargement of the lumen.

Group B.—Controls preserved at the start of the experimental period in Group B (5 months of age) show varying degrees of oviduct formation. Most ducts terminate at the level of the ovaries and sexual kidneys. Some even reach the lateral walls of the cloaca, where they end blindly. Figure 1, *a*, shows the oviducts in a control killed 3 weeks after the start of the experiment. The length of the two ducts often differs in the same animal. At completion of the experiment, all controls possess full-length oviducts which do not, however, open into the cloaca. They are slender structures, with a single layer of low columnar ciliated epithelium and narrow lumen. Figures 1, *b*, and 1, *c*, show the ducts as they appear in a gross view. Two animals have oviducts as in Figure 1, *c;* the remaining controls display a condition as in Figure 1, *b*, the ducts being thin and only slightly convoluted.

In many experimentals, the tips of the oviducts have not reached the cloaca by the end of the 10-week injection period. They end above the mesonephros in one individual, at anterior or middle gonad levels in a few, and at the posterior gonad level or even farther below this, alongside the lumbar kidney, in a larger number. In some larvae the ducts extend to the cloaca, and in a few of these even enter the cloaca by forming a narrow anastomosis with it. It is concluded that the final position of the oviducts in these hormone-treated animals is that which had been attained at the time that treatment was initiated.

Hypertrophy of the female sex ducts increases with the dosage of testosterone propionate. The characteristic range of stimulation with 0.01 mg. per week is shown in Figure 1, *d*, *e*, and *f; g* is typical of the effect of 0.05 mg. per week, and the condition obtained with 0.25 mg. per week appears in *h* and *i*. The oviduct exhibits a differential responsiveness along its length. The gonadal region is the first to be markedly stimulated by the hormone (this being apparent at the 0.01-mg. dose), the portion below the gonads responds next (at 0.05 mg.), and only with the 0.25-mg. dose does the anterior oviducal region above the ovaries reach a comparable degree of hypertrophy. Though the mesonephric ducts respond to the hormone earlier than do the oviducts, by the end of 10 weeks of treatment they are by far the smaller of the two. The final degree of oviduct hypertrophy is achieved within the last few weeks of the experiment. Animals preserved before this show less stimulation or none at all. Possibly an even more extensive growth might be attained with more prolonged treatment.

A surprising observation is made in experimental larvae with oviducts whose longitudinal growth was arrested above the cloaca. In most instances, the tip of the oviduct fuses with the mesonephric duct. This fusion is difficult to observe in gross dissections, as the gonoducts are enveloped by a common connective-tissue sheath. But study of sections clearly demonstratesthe existence of the anastomoses. The inserts in Figure 1, *d*, *f*, and *g* represent the mode of fusion in three such cases. Of the experimental animals with incomplete oviducts, 21 were studied microscopically, both right and left ducts being sectioned in 11 larvae and only one side sectioned in the

remaining 10. Among the 11 bilaterally sectioned individuals, 4 show fusion on both sides, 3 only on the left side (and have complete and separate ducts on the right), 3 on the right side only (2 of these have complete and separate ducts on the left and the other one, seen in Fig. 1, *d*, has an incomplete left oviduct, which terminates in a blind pocket without entering the mesonephric duct), and 1 has no fusion on either side (the right oviduct terminates blindly in the pronephric duct region, and the left duct reaches the cloaca). In 1 larva (Fig. 2, *b*) the left oviduct is completely absent. The 10 unilaterally sectioned individuals are all instances of fusion; the condition on the other side was not ascertained microscopically. These cases of joining of male and female gonoducts are distributed with equal frequency in all three dosage groups. Fusion occurs in 95.2 per cent (20 out of 21) of animals with at least one incomplete oviduct; 9.5 per cent (2 out of 21) of these larvae have at least one incomplete oviduct which terminates abruptly without fusion.

Histologically, a stimulated oviduct is hypertrophied primarily by increase and enlargement of the mucous glands in the thickened wall (usually in the oviducal region), or the wall remains a thin layer of ciliated low columnar or cuboidal cells and contains few such glands or none, while the lumen greatly enlarges (as in the uterine portion of the normal mature oviduct). In all cases in which the oviduct and mesonephric duct fuse, the stimulated oviduct is of the latter type. Figure 15 indicates the histological resemblance between oviduct and mesonephric duct (the latter is constructed of pseudostratified epithelium) in the fused region; there is, nevertheless, in this cross-section a sharp line of demarcation recognizable between the oviduct and mesonephric duct components where the histology of one abruptly ends and the other begins. Beyond the place of fusion there is always a gradual caudad tapering-off of oviduct tissue, until the single duct assumes exclusively the character of a mesonephric duct (into which mesonephric tubules enter). Mucous glands occasionally found in the upper regions of these oviducts may occur, for a short distance, in small scattered groups in the single mesonephric duct capsule below the place of fusion. This is presumably due to translocation of the glands rather than to induction of new ones. Figure 16 indicates such a case, in which mucous glands characteristic of normal mature Müllerian duct structure are found in the sheath of the mesonephric duct and actually enter the latter.

It has previously been stated that in hormone-treated females the oviduct is enlarged more than the pronephric and mesonephric ducts. In the event of oviduct absence, however, the uninhibited pronephric duct is much more highly stimulated. The same appears to be true of the size relationships between oviduct and mesonephric duct. Below a point of fusion of the two, the mesonephric duct, formerly much smaller in diameter than the oviduct, becomes greatly enlarged—as much so, in fact, as the oviduct above the point of fusion. These size relationships may be seen in Figure 1, *d*, *f*, and *g*.

Among controls, white animals have completely unpigmented sex ducts; dark larvae have melanophores distributed over part or all of the surface of the oviducts. This pigmentation is not influenced by hormone treatment. In cases of fusion of the oviduct with the mesonephric duct, the melanophores simply migrate over the transition point and continue into the single mesonephric duct below the place of fusion.

CLOACA

Normal *Ambystoma* females possess a smooth-surfaced cloaca, with small cloacal glands in the typical female pattern (Witschi, 1937). The cloacal glands of control males (Witschi, 1937) of comparable stage are enlarged, and the outer cloacal surface has numerous crypts, prominent rugae, and general hypertrophy of the cloacal lips. All controls in these experiments show the usual rudimentary female condition.

Group A.—The cloaca demonstrates little ability to respond to the male hormone during early larval life, when testosterone induces only a slight increase in the number of folds but no change in cloacal glands. This is true in spite of the fact that a marked external cloacal swelling occurs in these animals (partly visible in Fig. 3). The enlargement appears to be entirely of an edematous nature. Inception of the responsive period in cloacal development of both groups occurs at approximately $5\frac{1}{2}$–6 months after hatching. Following the untreated period, which started at the ages of 3–$4\frac{1}{2}$ months, Group A larvae again subjected to hormone administration at the ages of $8\frac{1}{2}$–10 months show stimulation of the cloaca and glands in the male direction, while their control sisters maintain the female condition. Continued treatment produces cloacal growth as in the male and equivalent (both with the 1 mg. per liter and with the 2 mg. per liter daily dose) to that obtained with 0.01 mg. per week in Group B by the injection method. No stimulation beyond this is achieved, even after 8 months of daily hormone additions to the water at these dosages.

Group B.—In Group B the highest dose (0.25 mg. per week) induces cloacal hypertrophy of such magnitude that the cloacal lips become everted and surrounded by many crypts and folds. The cloacal glands are greatly stimulated histologically. Less pronounced stimulation is obtained at the lower dosages. There is, in fact, such a marked differential in cloacal response that it is possible to judge the dose to which the animal has been subjected simply by a gross examination of the cloaca. As was the case with oviduct stimulation, the definitive cloacal response is not attained before the final weeks of the experiment. Figure 1 presents external views of controls and experimentals, indicating cloacal stimulation obtained with ten weekly injections of testosterone propionate at the three levels of hormone employed.

DISCUSSION

OVARIAL REACTION

In *Rana sylvatica*, 0.001 mg. of testosterone propionate per liter of water, given through the period of sex differentiation, is sufficient to produce a preponderance of males (Mintz and Witschi, 1946); up to 2 mg. per liter of the same hormone has no effect on developing urodele ovaries. The differentiated larval *R. clamitans* ovary is depressed and finally transformed to a testis in all cases with an amount of testosterone as small as 0.025 mg. per week (Mintz, Foote, and Witschi, 1945), while urodele females do not undergo sex reversal, and only 32.5 per cent suffer a partial cortical inhibition at doses up to ten times as great. Dantschakoff (1938) has proposed that the sex hormone of the heterogametic sex (e.g., the frog male and the salamander female) predominates and induces sex differentiation in that direction. But her contention is borne out neither by salamander parabiosis, by gonad-grafting experiments, nor by hormonal experiments with frogs.

Although testosterone propionate fails to induce testicular development in un-

differentiated genetic female salamanders or transformation of differentiated females to males, some cortical inhibition is observed among those females injected in later larval life, the incidence increasing with dosage. In studies of sex reversal in female *R. sylvatica* tadpoles following high-temperature application, Witschi (1929) observed that sex transformation did not begin with destruction of ovogonia or ovocytes but with suppression of further ovocyte formation. The same, though incomplete, reaction is observed in the cortical inhibition reported here. This initial picture of cortical suppression differs qualitatively from that obtained in large *R. clamitans* female larvae (Mintz, Foote, and Witschi, 1945), in which existing auxocytes undergo degeneration. It is suggested by Witschi (1929) that prevention of further formation of new ovocytes in the heat-treated tadpole ovary indicates that the female morphogenetic substance has been destroyed or its elaboration suspended. The same line of reasoning may be applied to the present results.

No measurements of rate of uptake, concentration, utilization, conjugation, or destruction of the hormone are available for a comparison of administration of hormone in the ambient water with the injection method. On the basis of degree of cloacal response, we are led to suggest that the dose of 2 mg. per liter of water given to young larvae is physiologically equivalent to an injection of 0.01 mg. per week in differentiated larvae. Therefore, we observe that (1) the ovarial cortex is not responsive to the amounts of hormone given during early larval life and (2) at a later age a responsive period is reached, but the physiological equivalent (0.01 mg. per week) of the previous highest dose (2 mg. per liter) now only constitutes a threshold for the reaction. Inasmuch as no animals receiving the hormone in the water, and only one receiving the 0.01-mg. injections underwent cortical inhibition, it appears that 0.01 mg. per week is a minimum for obtaining such inhibition and has no effect on most animals at that. This phenomenon becomes increasingly frequent at higher doses. Why, then, does not the anticipated compensatory medullary hypertrophy follow?

KIDNEY REACTION

Long before the stage or the dosage levels adequate for cortex inhibition are reached, an irreversible damage to the kidney has been initiated. Mesonephric degeneration is observed in very young animals treated shortly after hatching and continues to appear in a number of individuals treated at varying times thereafter by both methods and with all doses employed. The experimental animals have lost a great amount of mesonephric blastema. Though some blastemic cells are still present, these fail to multiply and subsequently to migrate into the gonad for the organization of the male rete, following reduction of the ovarial cortex. The absence of spontaneous medullary hypertrophy must therefore be regarded as the result of some loss in capacity of mesonephric blastema cells to divide under a depressing effect of male sex hormone. It is this failure of the proliferative source of medulla which is responsible for inability to undergo sex reversal. Vannini (1941) regards the mesonephric blastema as suprarenal, and the process of masculinization as involving hypertrophy of the suprarenal. Witschi (1942) has already pointed out that the mesonephric blastema does not simply represent adrenal cortical tissue but is an undifferentiated source giving rise to the latter, as well as to sex-gland medulla and to mesonephric tubules.

Dantschakoff (1937) describes precocious transformation of mesonephric tissue into epididymis, with a consequent fatal edema, in the bird embryo treated with testosterone propionate.

Apparently the anuran mesonephros, unlike that of the urodele, is not noticeably affected by testosterone. Wolf (1940) has reported hypertrophy and encapsulation of kidney tubules, and kidney hyperemia in adult *R. pipiens* receiving testosterone propionate or estradiol benzoate. In this case, however, the injected testosterone dose was very high.

According to Korenchevsky and Ross (1940), male sex hormone has a beneficial, or "nephrotrophic," effect upon the kidneys of rats, causing hypertrophy. Slight pathological changes are produced only when the androgen is employed in large doses. These changes resemble those which they ordinarily obtained with estrogens, which were toxic rather than nephrotrophic and caused cystic kidneys with marked tubular atrophy and dilation.

Foote (1941) found kidney changes with androgens but little effect with estrogens. He interprets as stimulation the presence of distended, fluid-filled mesonephric tubules in his testosterone-treated salamanders, but present observations of more extensive kidney alterations would appear to indicate that the hormone exerts a depressing or destructive effect. Adams (1946) has recently described production of cystic mesonephric bodies with dilated tubules in adult male newts upon administration of the synthetic estrogenic compound, diethylstilbestrol.

Accumulation of fluid in the body cavities of many treated animals is probably a combined result of impaired kidney function and stasis of fluid in the enlarged mesonephric ducts (Figs. 3 and 5).

AMBIGENIC NATURE OF TESTOSTERONE PROPIONATE

Previous observations of kidney inhibition with sex hormones (Korenchevsky and Ross, 1940; Adams, 1946) suggest that this phenomenon is characteristically obtained with estrogens rather than with androgens. The hypothesis is therefore now advanced that the similar effect observed in *Ambystoma* with testosterone propionate treatment is fundamentally a gynogenic action of the hormone, particularly in its influence on the mesonephric blastema. This is consistent with the fact that gynogens cause female differentiation of genetic males by suppressing the proliferative capacity of the medullary elements. The action of testosterone on the primary sex characters of salamanders is therefore both gynogenic and androgenic, seen by medullary suppression and cortical inhibition, respectively. Kidney damage precedes cortical inhibition, however, appearing at an earlier stage in development and with lower levels of hormone. Therefore we may regard the hormone as prevailingly gynogenic.

The presence of testicular ovocytes in testosterone-treated genetic male larvae and a shift of the sex ratio in the female direction have been offered by Foote (1941) as evidence of the feminizing action of this steroid.

Simultaneous stimulation of male and female secondary sex characters is further indication of the ambigenic nature of testosterone propionate, although greater hypertrophy of the female gonoducts testifies to the prevailing gynogenic effect.

Numerous investigations (reviewed by Rodgers and Risley, 1938) have indicated that sexually dimorphic development of secondary sex characters in urodeles occurs under the influence of hormones released by the gonads. Normal

oviduct hypertrophy is governed by an ovarian hormone and correlated with the presence of many large-yolked ovocytes and those actively forming yolk. None of our animals show active yolk deposition. Manifestation of the gynogenic nature of testosterone propionate in its stimulation of the differentiated urodele female sex ducts has previously been observed in *Ambystoma* by Foote (1941), in *Triturus viridescens* by Adams, Gay, and Terzian (1941), in *Triton cristatus* by Galgano (1942), and by various workers in other vertebrates (review of literature in Adams, 1946). The data may be interpreted to mean that testosterone propionate is not the same as the mature urodele male sex hormone. Stimulation of the oviducts with testosterone propionate has also been observed in adult anurans, including *R. pipiens* (Puckett, 1939; Wolf, 1939 and 1940), *Acris gryllus* (Greenberg, 1942), *Bufo americanus* (Puckett, 1939), and *B. fowleri* (Blair, 1942). The underlying nature of this heterotypic gynogenic response remains obscure. The combined observations on urodeles cover a wide range of dosage, and it is now apparent that the effect is obtained at low, as well as at high, levels. Castration experiments, combined with testosterone treatment, have been performed in other species to study the possible role of the gonads in the oviduct growth. Wolf (1939) observed enlargement of the oviducts of adult *R. pipiens* males under such conditions, although she reports no studies of hormone-treated normals. Adams, Gay, and Terzian (1941) obtained oviduct hypertrophy in the newt with large doses of testosterone propionate, and their figures on relative weights of the female gonoducts of normal and castrate control and androgen-treated adults suggest that the oviduct response is in part mediated by the ovary, since the ducts of injected castrates were less hypertrophied than were those of injected normals. In female starlings, Witschi and Fugo (1940) demonstrated that the oviducts of castrates are stimulated by testosterone as much as those of normals are, thus indicating the direct gynogenic, and therefore, "paradoxical," action of this hormone on the Müllerian ducts. It has also been suggested that the oviduct response is mediated by pituitary stimulation which causes an increased output of estrogen by the ovary. De Allende (1939) found a direct action of implanted pituitaries on the secretory activity of oviducts in ovariectomized *B. arenarum*. Testosterone propionate injections in normal toads were negative.

COMPARISON WITH GRAFTING AND PARABIOSIS

Testosterone propionate treatment does not duplicate the action of a graft testis in inducing reversal of the ovary. Humphrey (personal communication) has found that animals from the same spawning as those in Group A, Series II, which received graft testes, show reversal of ovary to testis at the age of 2 years. Since half of these presumably were WW and half ZW, reversed animals of the WW genotype would be included, although the results of breeding experiments to establish this conclusively are not yet available.

In heterosexual salamander parabionts, the male's testis produces some substance which completely inhibits cortical differentiation in the female cotwin, eventually resulting in development in the male direction. This substance however, lacks any pronounced effect on the female's gonoducts (Witschi, 1940).

It therefore appears that the male sex hormone employed in the present experi-

ments is not identical with the male inductor substance.

"CONTACT INHIBITION" OF NEPHRIC DUCTS BY OVIDUCT

Foote (1941) and Witschi (1942) describe stimulation of the pronephric ducts far in excess of that obtained here. One of the factors responsible for the divergent results is apparently that of species differences in time of responsiveness to testosterone. Another is the presence or absence of the oviduct. At the age when the pronephric ducts in the species studied by Foote started to show stimulation with androgen, the oviducts were not yet present. Similarly, in Witschi's material, the downgrowth of the oviducts had been arrested in the pronephric body region. The pronephric ducts, then, are very highly stimulated when the oviducts are absent or restricted to the level of the pronephric bodies. When the oviducts are present, however, as in *A. mexicanum*, these female ducts are greatly hypertrophied by the male hormone, and the pronephric ducts (if still present) show relatively little stimulation. These facts, combined with the bilaterally asymmetrical case already described (Fig. 2, *b*), suggest the existence of some sort of inhibition of the pronephric duct by the corresponding oviduct. This inhibition must be of a contact nature (probably chemical), since a stimulated oviduct on one side is accompanied by a small pronephric duct, while on the other side of the same animal, where the oviduct is absent, the pronephric duct is greatly stimulated.

The same competitive situation appears in the relationship between the oviduct and the mesonephric duct. When both coexist, the oviduct takes up most of the stimulation which we may say is "jointly allotted" to the two ducts by the hormone. But when an oviduct fuses with a mesonephric duct, the single mesonephric duct below the point of fusion exhibits the degree of stimulation shown by the oviduct at the level in question in animals in which the two ducts remain separate and do not fuse (Fig. 1).

Humphrey (1941) has discussed a similar finding on size relationships of Wolffian and Müllerian ducts in experimental *Ambystoma* hermaphrodites, in which the female duct near the ovary is enlarged while the testis graft has stimulated development of the male duct in the lumbar region. He points out that the two ducts are never both maximally developed in any given region; as one increases in diameter, the other decreases, and the change is often abrupt. When parts of the Müllerian duct disappear, the Wolffian duct is larger than elsewhere. He speaks, therefore, of the "apparent inhibitory effects of one duct upon the closely associated portion of the other." (These animals possessed no pronephric ducts.) Adams (1938, 1946) has also described a difference in degree of reaction in two regions of the oviduct in *Triturus*, the anterior part (where there is no Wolffian duct) being of greater size than the segment along the mesonephric kidneys.

SEMINAL VESICLES

The presence of "seminal vesicles" on the lower portions of the stimulated mesonephric ducts of two experimental axolotls, a species not normally possessing such structures, resembles the same occurrence reported in one testosterone propionate–injected male *R. clamitans* larva (Mintz, Foote, and Witschi, 1945) and also recalls Witschi's observation (1942) of pouches and crypts covering the surface of highly stimulated pronephric and mesonephric ducts in *A. tigrinum* females, as well as the one case

of the similarly stimulated left pronephric duct reported in this paper.

ARREST OF OVIDUCT DOWNGROWTH; GONODUCT FUSIONS

Cessation of the downgrowth of the oviducts in the presence of male sex hormone indicates (as pointed out by Witschi, 1942) that the blastemic cells of the inductive center at the lower end of the growing ducts have been destroyed or their formation halted. The presence of such a sensitive inductive center has been verified by tube-inductor extirpation experiments (Riley, 1934). Probably the downgrowth of the oviduct is not stopped at the exact time that hormone treatment is started. (The reader will also recall that oviduct hypertrophy under the influence of the androgen does not begin immediately.) It is unlikely that the oviduct was in contact with the cloacal wall when testosterone was first given to animals which eventually showed oviducts entering the cloacal opening. How long the growth of the oviduct can still continue under the influence of sex hormone is not yet known. The fact that the hormone causes precocious opening of the oviduct into the cloaca—a phenomenon which does not normally occur until sexual maturity—suggests that the normal process may depend upon the influence of female sex hormone (possibly controlled by the pituitary gland) rather than upon mechanical pressure from eggs in the oviduct, as has been alternatively proposed.

It is at present believed (outlined in Witschi, 1942) that in amphibians the oviduct arises from a peritoneal area (lateral mesoderm) adjacent to the pronephric body, while the mesonephric duct is formed separately and independently from intermediate mesoderm. It has, however, been often claimed in the past that the oviduct develops embryologically by splitting off from the Wolffian duct and that the funnel differentiates from the pronephros. This theory was first advanced for urodeles by Fürbringer in 1878, and similar interpretations are reviewed by Hall (1904). In view of the fact that no recent work has been done to clarify this situation in elasmobranchs, the above mode of differentiation is still considered applicable to that group of vertebrates. The present observations of gonoduct fusions cannot be regarded as necessarily bringing corroboration to bear upon the concept of oviduct origin from the segmental (pronephric and mesonephric) ducts, and an understanding of its significance will depend upon further investigations. The oviduct's entrance into the mesonephric duct may be thought of as a precocious nonspecific opening of the female duct, analogous to its premature entrance into the cloaca when it has reached the latter before hormone is administered.

If we conclude that the mesonephric duct produces some substance which passes into the oviduct via the common lumen and thereby inhibits oviduct glandular development, we must assume that the fusion occurs before the hormone has effected oviduct hypertrophy.

SUMMARY

Ambystoma mexicanum and *A. mexicanum-tigrinum* hybrid larvae of exclusively female genetic constitution (ZW and WW) were treated with testosterone propionate. A total of 295 animals (including controls) were raised in water containing 0.02, 0.1, or 0.5 mg. of the hormone per liter, starting at 15, 54, or 63 days after hatching. In a second group, 166 older, differentiated larvae (5 months of age) were injected over 10 weeks with 0.01, 0.05, or 0.25 mg. per week (also including controls).

None of the animals underwent sex re-

versal, and no intersexes were found. Among the second group, 1 animal (2.5 per cent) of those receiving the 0.01-mg. dose, 5 (12.5 per cent) treated with 0.05 mg., and 13 (32.5 per cent) at the 0.25-mg. level underwent cortical inhibition, involving prevention of formation of new ovocytes rather than degeneration of existing ones. This was correlated with the occurrence of varying degrees of damage to the mesonephros, including reduction in number of blastema cells. Kidney depletion was also observed among early treated larvae as well as many of the late treated ones not exhibiting ovarial reduction. It therefore appears that the mesonephros takes priority over the ovary in responding to testosterone, and when a dose producing inhibition of the gonadal cortex is reached (the first step toward sex reversal), the animal no longer possesses a mesonephric blastema capable of proliferating and supplying the rete testis elements requisite for medullary growth and true sex reversal.

The hormone partly prevents normal pronephric duct regression. These ducts are stimulated, but less so than the mesonephric ducts. Experimental evidence suggests a contact inhibition of the nephric ducts by the oviducts, whereby the former are maximally stimulated only in the absence of the latter. Oviduct downgrowth is arrested, but existing portions are hypertrophied to a far greater extent than are the mesonephric ducts. Female sex ducts which have attained full length usually open precociously into the cloaca. In most individuals with incompletely formed oviducts, the lower end of the duct opens into the mesonephric duct. Two larvae (in the younger group) had seminal vesicle-like structures on the lower parts of the stimulated mesonephric ducts.

The cloaca assumes the male type, and urinary collecting ducts at the level of the lumbar kidney migrate posteriorly, approximating the male pattern.

Evidence is discussed indicating that testosterone propionate (1) is an ambigenic hormone, but prevailingly gynogenic to urodeles; (2) is not the same as any of the urodele embryonic sex inductor substances; and (3) is not the same as the adult urodele male sex hormone.

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Animals shown in Figures 3–8 received early hormone treatment (Group A); those in Figures 9–16 received treatment in later larval life (Group B).

PLATE I

FIG. 3.—Animal treated with 0.02 mg. of testosterone propionate per liter of aquarium water per day for 72 days, beginning at 63 days after hatching. The body cavity is distended with fluid. ×2.1.

FIG. 4.—Ovaries, mesonephric kidney, and mesonephric duct of 110-day-old control larva. Note absence of oviduct. ×200.

FIG. 5.—Ovaries and kidneys of the same animal as in Fig. 3. Enlargement of mesonephric tubules and loss of intertubular tissue. Gonad growth retarded. ×200.

FIG. 6.—Stimulated mesonephric ducts just above the cloaca in 116-day-old female raised in 0.02 mg. per liter per day of testosterone propionate, starting at 54 days after hatching. The duct on the right has small budlike structures resembling seminal vesicles. ×100.

FIG. 7.—Complete absence of mesonephric tissue (other than peritoneum and stroma) at the lower level of the gonads in a larva 10 months of age, which received the 0.1-mg. dose of hormone for 2½ months in early larval life, followed by a 5½-month period without treat-

PLATE I

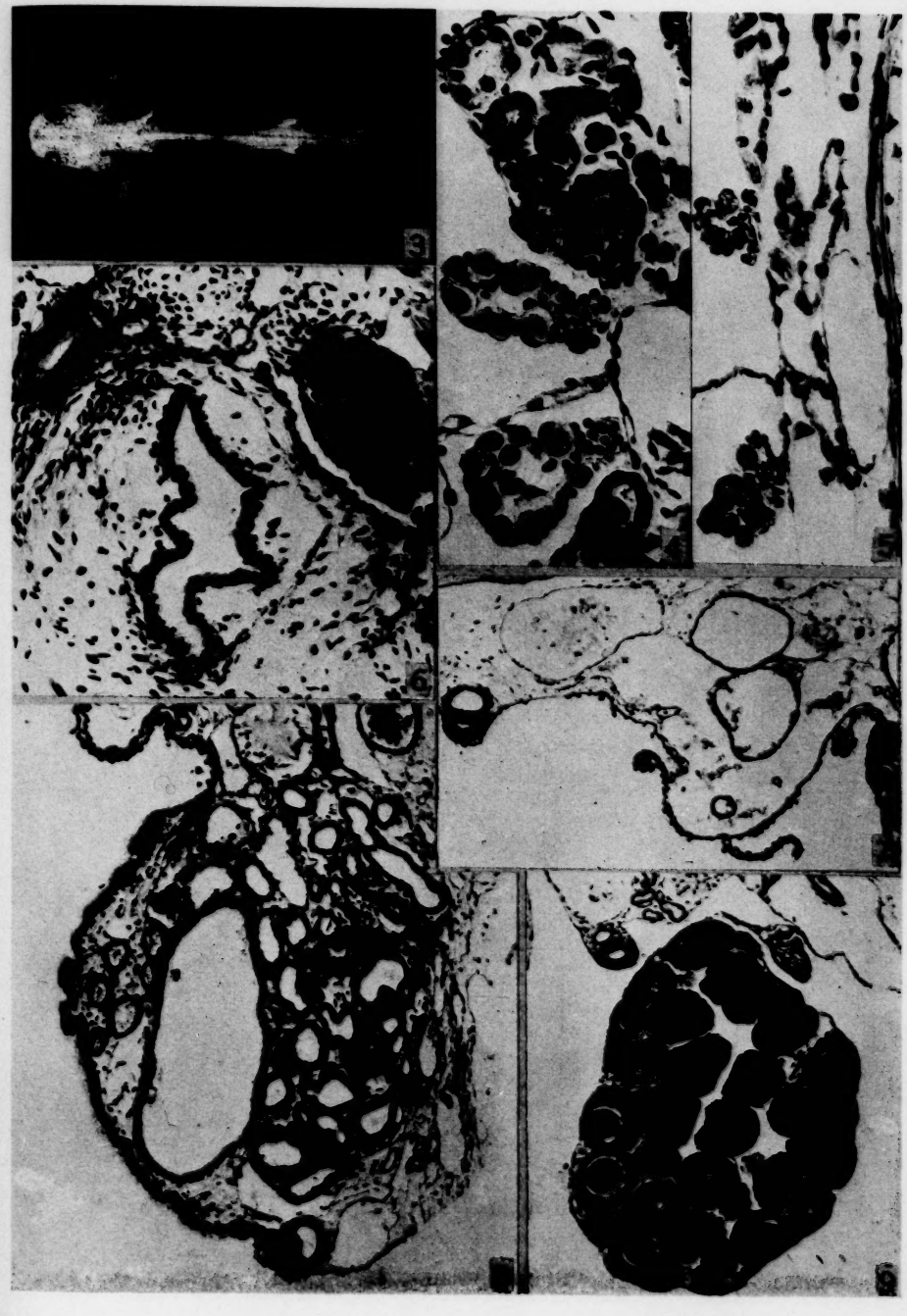
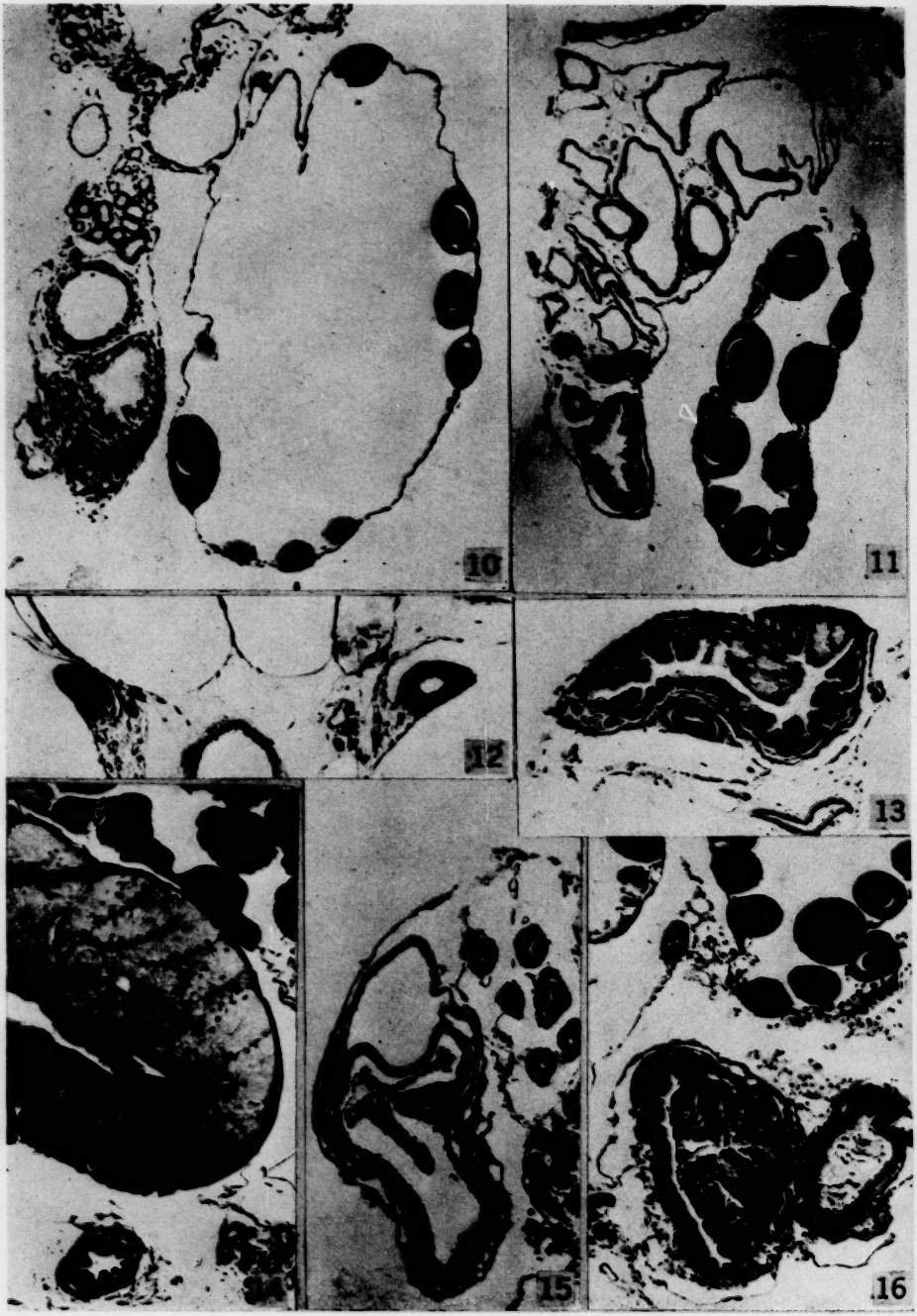


PLATE II



ment. Unstimulated right oviduct and mesonephric duct. ×50.

FIG. 8.—Same animal as in Fig. 7. Left mesonephric body posterior to the region shown in Fig. 7. Enormous enlargement of tubules; spread of heavy ciliated epithelium over the peritoneal surface. ×50.

FIG. 9.—Right ovary, oviduct, and mesonephric duct (with mesonephric tubule entering it) of an uninjected control animal preserved at the end of the 10-week experiment on older larvae. ×50.

PLATE II

FIG. 10.—Female injected for 10 weeks with 0.01 mg. per week of testosterone propionate. Ovary (right side) showing extreme cortical inhibition, with only a few scattered auxocytes and enlarged ovarial sac cavity. Stimulated oviduct and mesonephric duct (mesonephric tubule entering mesonephric duct). ×50.

FIG. 11.—Right ovary, kidney, and gonoducts of an experimental larva which received the dose of 0.05 mg. per week of testosterone propionate. Note ovarial inhibition and stimulated sex ducts. The mesonephros contains few blastema cells, and the tubules are much inflated. ×50.

FIG. 12.—Small oviducts anterior to the ovaries in a control female at the end of the experiment. Complete absence of pronephric ducts. ×100.

FIG. 13.—Greatly hypertrophied oviduct (right side) and slightly stimulated pronephric duct anterior to the sex glands after treatment with the 0.25-mg. dose of hormone. ×50.

FIG. 14.—Part of stimulated left oviduct (*center*) and mesonephric duct (*below*), following injection with 0.25 mg. of hormone for 10 weeks. Inhibited ovary and few remaining tubules of mesonephros can be seen. ×50.

FIG. 15.—Fusion of left oviduct (*lower*) and mesonephric duct (*upper component*) in the lumbar kidney region of an experimental female (0.01-mg. dose). See Fig. 1, *f*. ×50.

FIG. 16.—Right mesonephric duct of treated female (0.25 mg. per week) several millimeters below where the oviduct has fused with the mesonephric duct. Mucous glands characteristic of mature oviducal structure are entering the mesonephric duct. A loop of the mesonephric duct appears to the right, and part of the inhibited ovary is visible. ×50.

THE EFFECT OF MALE HORMONE ON AGGRESSIVE BEHAVIOR IN MICE[1]

ELIZABETH A. BEEMAN

MALE mice are extremely combative and, when caged together, usually establish social hierarchies based on dominance-subordination relationships. Evidence that aggressive behavior among such groups of mice may be very pronounced is found in the fact that deaths from active fighting have been frequently reported (Crew and Mirskaia, 1931; Greenwood *et al.*, 1936; Retzlaff, 1938).

Seven types of social orders have been described by Uhrich (1938) for laboratory groups of five albino mice; six of these are based on fighting. If the social orders representing varying degrees of stability are combined and if one in which no fighting occurs is eliminated, two main types result: a pyramidal order, and the type most frequently found, in which there is exclusive dominance by one mouse and no fighting or resistance

[1] I wish to express my deep appreciation to Dr. W. C. Allee for constructive criticism and interest given during the course of this investigation, and to the Jackson Memorial Laboratory and Dr. J. P. Scott, director of behavior studies, for animals, research facilities, and help extended to me during the summer of 1946. I also wish to thank Dr. Sewall Wright for critical aid with statistics.

by the four subordinates. These social orders range in permanence from 1 or 2 days to several months.

Several factors have been shown to be of importance in determining social dominance in mice. Uhrich (1940) suggests that if the difference between the body weight of two mice is greater than 5 gm. (15–20 per cent of total weight), the heavier mouse will tend to win encounters. Fatigue, illness, and severe injuries reduce aggressiveness in most mice (Ginsburg and Allee, 1942). Under the experimental conditions used by these authors, starvation for 12, 24, or 36 hours did not induce changes in aggressive behavior; and, after 6–8 hours' deprivation of water, access to a single water bottle with a small nozzle which permitted only one mouse to drink at a time did not stimulate fighting. Among the mice used by Uhrich (1940), relative age appears to have been of no importance in the winning of encounters, provided that the weight differences were not too great.

It has been demonstrated by Ginsburg and Allee (1942) that previous experience is a very important factor in determining social dominance. They have shown that, as a result of numerous defeats, mice may be conditioned to be less aggressive and can also be made more aggressive by a series of victories over submissive mice. Conditioning of either type affects the ability of a mouse to secure or to maintain high social status. Among mice, previous experience in winning encounters is apparently a factor of greater importance in maintaining social dominance than is physical well-being. Beeman and Allee (1945) report that C57 black mice in extreme stages of vitamin B_1 avitaminosis will maintain high social rank despite physical weakness sufficient for almost complete impairment of active aggressiveness.

Scott (1940, 1942), in studies of prefighting behavior in mice, described differences in aggressiveness between genetically distinct strains of mice at the Jackson Memorial Laboratory. In addition to the hereditary differences in prefighting behavior described by Scott, Ginsburg and Allee (1942) have demonstrated differences in aggressiveness, including fighting ability, for the C57 black, C3H, and Bagg albino strains of mice. Mice of these strains have been inbred for many generations, and each strain is almost completely homozygotic. After observing many battles, Ginsburg and Allee were able to establish relatively stable hierarchies, which varied in the middle ranks and were most stable at the extremes.

The data reported by Scott (1942) and by Ginsburg and Allee (1942) are primarily concerned with the ability of mice to begin or to win interstrain or intrastrain encounters. The normal aggressive behavior of mice (see p. 378) has not been described in terms of the frequency with which various components of the aggressive pattern occur within a given strain or in terms of the relative aggressiveness within strains.

Although numerous studies have demonstrated the importance of male hormone in stimulating aggressive behavior among vertebrates in general, there is little direct evidence concerning the effect of androgens on aggression in rodents. Seward (1945*a*) reports unpublished data by Riege, who found that after castration three male rats were reduced to the level of the controls, although they had previously scored reliably above the others in aggressiveness. Treatment with testosterone propionate significantly raised their aggressiveness. Seward states (p. 194) that ". . . . it appears likely that androgenic hormone

plays a definite part in activating aggressive behavior in male rats."

Beach (1945) castrated a male rat that was "an unusually vigorous copulator when placed with females in heat." Masculine copulatory responses were not significantly altered when first tested, 6 days after castration. The second test, conducted 5 days later, showed loss of both copulatory behavior and aggressiveness. Injections of testosterone propionate restored copulation, including the power of ejaculation, and revived aggressiveness.

The only study which bears directly on this problem in mice is that by Uhrich (1938), in which it was shown that castration of albino mice results in a considerable diminution of the fighting reaction of some males, but it does not completely inhibit the reaction in all individuals.

The present study was undertaken in an attempt to describe more fully, and in quantitative terms, the normal aggressive behavior of male C57 black and Bagg albino mice and to investigate further the role played by androgens in such behavior.

ANIMALS AND THEIR TREATMENT

All the mice used in this investigation were C57 black or Bagg albino males obtained from the stocks of mice at the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, or from a mouse colony maintained at Whitman Laboratory, University of Chicago. The animals in the latter colony are descended from mice originally obtained from the inbred stocks at the Jackson Laboratory and are members of the stocks used by Ginsburg and Allee (1942) and by Beeman and Allee (1945).

Experiments were designed to yield additional information of the characteristics of normal aggressive behavior of C57 black and Bagg albino male mice in intrastrain encounters and, in addition, to investigate the role which male hormone plays in determining aggressive behavior. The mice used included thirty-seven C57 black mice (ten experimental sets), and thirty-one Bagg albino mice (eight experimental sets). The experiments on these sixty-eight mice, which will henceforth be referred to as "B" mice, were performed at the Jackson Laboratory and were primarily concerned with the relation of male hormone to aggressive behavior. Additional experiments were run on six sets of four normal C57 black mice and three sets of four normal Bagg albino mice. These mice, designated as "W" mice, were used in experiments carried out at Whitman Laboratory in Chicago.

B MICE

At Chicago, the mice were bred and isolated in sheet-metal cages that measured $7 \times 7\frac{1}{4} \times 12$ inches. At the Jackson Laboratory each mouse was isolated in a wooden box which measured $11\frac{1}{2} \times 5\frac{1}{4} \times 6$ inches and which had a wire-mesh cover with a food hopper, so that Fox Chow Checkers were constantly available to the mice, in addition to water. Shavings were placed in the wooden cages in which the mice remained isolated except during the staged encounters. They were transferred to clean boxes every 2 weeks by picking up by the tail by hand, were handled only by the author and only at this time. Eleven mice of each strain were shipped from the colony at Chicago to Bar Harbor in a special box, which kept each mouse isolated from every other mouse. Judged by their subsequent behavior, the shipping did not affect their aggressiveness.

Age at initial isolation in the eight sets of albino and ten sets of C57 black mice varied from 21 to 64 days of age. All the

mice in one set were isolated at the same time and were littermates or from litters born a few days apart. Each mouse was earmarked for purposes of identification.

Following the procedures used by Ginsburg and Allee (1942) and Beeman and Allee (1945), round-robins of encounters were staged, in which each mouse met every other mouse in the experimental group. Thus in a group of four mice there were six encounters per round. During a week, the mice in each experimental group met in one complete round-robin on each of 2 days. The order of encounters was varied, so that individuals did not meet in the same sequence in consecutive rounds. The animals which were brought together in round-robin encounters were considered members of the same "group" or "set" of mice, regardless of the fact that during the greater part of the time they were not caged together.

Encounters were staged in a neutral wire cage (mesh $\frac{5}{16}$ inch). This cage, which had a removable sheet-metal bottom, measured $9\frac{1}{2} \times 9\frac{1}{2} \times 12$ inches and was divided into two equal sections by a galvanized-iron partition that could be readily raised. In order to transfer a mouse from his home cage to the "fighting cage," a piece of wire screen was placed in the home cage; after the mouse had climbed onto the screen, it was transferred to the neutral cage, where it remained until the mouse had climbed off. The same procedure was used to return a mouse to his home cage.

During an encounter all room lights were turned off, and a lamp with a 60-watt bulb was placed over the cage. Encounters were begun by raising the partition between the two mice and were permitted to continue until one of the two had won the encounter or, if no decision was reached, until 5 minutes had elapsed. An encounter is considered won when one mouse actively submits to another or squeaks and runs away.

Six sets of castrate albino mice and seven sets of castrate C57 black male mice were each allowed to experience at least six rounds of encounters. Of the twenty-three albino mice castrated, one set of three mice was deprived of its gonads at 27 days of age, and five groups of four mice were castrated at 29, 44, 77, 80, and 83 days of age, respectively. Age at isolation varied from 21 to 54 days after birth in the six groups.

Twenty-five C57 black mice were divided into four groups of four mice each, which were castrated at 23, 54, 74, and 83 days, and three groups of three mice, which were castrated at 27, 62, and 90 days of age. Age at isolation varied from 23 to 61 days after birth. The first staged encounters among the thirteen sets of castrates occurred from 25 to 140 days after castration.

A control operation duplicating castration procedures was performed on one set of four black mice and one set of four albino mice. In this operation the mouse was anesthetized, the abdomen was opened, and the gonads were pulled out through the incision and returned to the abdominal cavity. The animal was then sewed up. One normal group of four albino mice and two normal groups of four black mice were used as additional controls.

After six rounds of encounters had occurred, pellets of testosterone propionate[2] were implanted subcutaneously in three of the six sets of castrate albino mice and in four of the seven sets of castrate C57 black mice. The average weight of the eleven testosterone pellets implanted in the albino mice was 15.1

[2] The pellets of testosterone propionate were made available through the courtesy of Dr. Erwin Schwenk of the Schering Corporation.

mg. (range 14.5–15.6). The fourteen pellets placed under the skin of the C57 castrates averaged 15.5 mg.; they ranged in weight from 15 to 16.2 mg. The disk-shaped pellets, which were weighed to 0.1 mg. on an analytical balance, had a diameter of 4 mm. and a thickness of 1.25 mm.; the average surface area was 40.8 sq. mm.

Six pellets were recovered, upon which rates of absorption of the testosterone propionate could be approximated. The method of calculation consisted of dividing the total amount of hormone absorbed by the number of days during which the pellets were implanted. For these pellets the rate was found to be 0.15 mg. per day. Kochakian (1941) states that the amount of hormone absorbed is in relation to the surface area of the pellet and not to the mass. He implanted mice with pellets of testosterone propionate of two sizes, 21.8–24.1 mg., average surface area 44.8 sq. mm., and 9.8–12.6 mg., average surface area 27.3 sq. mm. From his data we can calculate that the absorption rate for eight of the larger pellets was 0.183 mg. per day and for three of the smaller pellets, 0.123 mg. per day. These figures are in line with those reported here, since the pellets used to compute absorption rates ranged in weight from 15 to 16 mg. and had a surface area of approximately 41 sq. mm.

In order to implant the pellets, the mice were etherized, most of the hair was removed from an area over the right shoulder, and, after the region had been moistened with alcohol, a cut was made through the skin. A pocket in which to place the pellet was opened out, with the aid of forceps, between the skin and the underlying muscles. After the pellet had been inserted, the incision was sewed up. The pellets were allowed to remain under the skin until six additional rounds of encounters had been completed by the seven sets of castrate mice during a period of 3 weeks.

The implanted pellets caused local damage to the tissues and made the overlying skin very soft. During the fights which began soon after the pellets were introduced, the skin in this area was frequently torn, and the pellets protruded from the wound. In such cases the mice were again anesthetized, the pellets returned to the pocket, and the skin sewed. In instances in which the pellet was lost after the skin had been torn, a second pellet was introduced. A few pellets were placed beneath the skin in the region of the right hip. However, this site was as exposed to injury during an attack as the right shoulder, and the pellets did not always remain under the skin in this area.

After the second six rounds of encounters, the mice were again anesthetized, and the pellets were removed, washed in distilled water, and, after being dried, were weighed. Two sets of C57 black mice and one set of albino mice from which the pellets had been removed experienced a third group of six additional rounds of encounters—a total of eighteen rounds per set. Only mice in which the fate of all implanted pellets was known were allowed to continue in this third group of six rounds of encounters. If, at the end of the second group of six rounds, a pellet could not be recovered, that particular mouse was excluded from further encounters.

In addition, two sets of castrate albino mice and two sets of C57 black mice received subcutaneous implants of pellets of dextrose after the first six rounds. These pellets were of approximately the same size as those of testosterone propionate and were similarly implanted. The average weight of the eight dextrose pellets implanted in the albino mice was 13.2 mg. (range 10.5–17.8), and that of

seven pellets given to C57 castrates was 14.7 mg. (range 12–19.2 mg.).

W MICE

In Chicago the mice were kept in a dark room in Whitman Laboratory. The ceiling lights were on for various lengths of time during the day, and the temperature was fairly constant, approximately 23° C. during the winter months and 25° C. during the summer. The temperature infrequently varied between 20° and 30° C., with no observable effect on the aggressiveness of the mice.

Breeding and isolation of the mice occurred in screen and sheet-metal cages whose measurements were $7\times7\frac{1}{4}\times12$ inches. The mice were transferred to larger wire-meshed cages, $9\frac{1}{2}\times9\frac{1}{2}\times7$ inches, a few days before staged encounters were to begin and remained isolated in these cages except during the encounters.

The cage and experimental procedures used for staging encounters were identical with those already described for the B mice. In all instances two round-robins were completed each week. However, in some sets, all the encounters of one round-robin were not run on one day. Consequently, any given mouse in these sets experienced fewer encounters per day than did the other mice, although the total number during a single week was the same in both cases.

All the mice were isolated as soon after 21 days of age as cage space was available. Average age at isolation of five groups of black mice and three groups of albinos was 28.5 days.

RESULTS

BEHAVIOR OF NORMAL MICE IN INTRA-STRAIN ENCOUNTERS

The normal aggressive behavior pattern of adult, male, C57 black and Bagg albino mice may include hair-fluffing, tail-rattling, mincing, parrying, attacking, chasing, and fighting. All these terms except parrying are defined by Ginsburg and Allee (1942) or by Beeman and Allee (1945). For example, while mincing, a mouse rapidly takes many small steps near or in circles around another mouse. While parrying, mice balance on their hind legs and strike at each other with their forelegs. In addition to the behavior mentioned, normal adult males, when caged together, may spend time in grooming or sniffing each other, independently investigating the cage, actively submitting, squeaking, and running away. Because, in many cases, it was impossible to distinguish between grooming and sniffing, such activities have been combined and classified as "grooming."

After the control operation, mice of both strains behaved very much like normal animals. Since some groups of mice experienced more rounds of encounters than other groups and a comparison of total amounts of activity exhibited would not be meaningful, it was necessary to compute the average activity per group per round in order to make comparisons of the behavior of the operated and the unoperated mice. Except for one instance in which the operated-normal albino mice gave 23 more tail rattles than the largest number given in any one round by normal albino mice, the activity of the operated controls of both strains was within the general range of variability of the various sets of normal controls. In both strains the greatest number of attacks or fights during a single round occurred in normal sets. An analysis of the behavior of one group of normal albino mice and that of one group of operated-normal mice of this strain is given in the first two horizontal columns in Table 1. It is evident that the aggressive behavior of the operated mice was not significantly depressed. Actually,

when comparisons are made on the activity observed in the same number of rounds, the operated albino mice exhibited, on the average, more tail-rattling, mincing, attacking, and fighting per round than did the normal albino mice. The difference between the amount of attacking, parrying, and fighting displayed by normal and operated-normal mice is very slight.

In the third and fourth horizontal columns of Table 1 it can be seen that durmade between the behavior of these two groups.

The various components of the aggressive behavior pattern may not be exhibited in maximum amount during the first few encounters, and in later encounters fluctuations in the frequency with which these behavior signs occur is evident. In two groups, each of which was composed of four normal males, black Sets VI and VII, the only activity exhibited during the first round of

TABLE 1

COMPARISON OF THE BEHAVIOR OF NORMAL MICE WITH THAT OF NORMAL ANIMALS UPON WHICH A CONTROL OPERATION HAS BEEN PERFORMED (B MICE)

Mice	Total No. Rounds per Group	Total No. Encounters per Group	Average Activity per Group per Round				
			Tail Rattle	Parry	Mince	Attack	Fight
Bagg albino: 1 group of 4 normals	12	72	26.5	0.085	1.6	14.2	8.5
Bagg albino: 1 group of 4 operated normals	12	72	32.5	0.083	4.7	15.4	8.8
C57 black: Average of 2 groups of 4 normals	6	36	4.6	1.8	1.8	24.4	15.6
C57 black: 1 group of 4 operated normals	6	36	4.5	2.0	8.1	24.3	21.5

ing six rounds of encounters the normal C57 black mice displayed practically the same amount of tail-rattling, parrying, and attacking per group per round as did the operated animals of this strain. On the other hand, the latter group more frequently engaged in mincing and fighting. Consequently, since the control operation had no observable depressing effect on the behavior of either the C57 black or Bagg albino mice and since the activity of the control-operated mice was within the range of variability of the normal controls, data from normal and operated-normal mice have been combined, and no further distinctions will be

encounters was grooming or sniffing and independent activity. In a similar group —black Set III—the only aggressive behavior in the first round of encounters was one tail rattle. During the second round of encounters all types of aggressive behavior were present in black Sets III and VII and continued to appear, with fluctuations in frequency, until the end of the 18th and 16th rounds, respectively, when the encounters were discontinued. The four mice of black Set VI were a little slower in beginning to act aggressively. Only 2 attacks and 1 parry were made in the second round, but in the third round the complete ag-

gressive pattern appeared. All three groups were similar in showing some aggressive activity in the second round of encounters and in continuing to act aggressively until the termination of the experiments.

In all graphs in this report, a scale of activity has been adopted in which the occurrence of 1 tail rattle, attack, fight, or other behavior sign has arbitrarily been made equal in value. Thus comparisons of the relative frequency with which various behavior signs occur may be made.

Figure 1 indicates the amounts of grooming, tail-rattling, attacking, and fighting which occurred during several rounds of encounters in two groups of normal C57 black mice. These graphs clearly show the fluctuations that may normally appear in the number of times that a given bit of aggressive behavior may be given in a succession of rounds of encounters. They also show that the increases and decreases in amount of a given aggressive act do not occur at the same time in different groups of mice. Attacking and fighting began during the second round of black Set VII (Fig. 1, *a*), and the first major rise in frequency of attacking and fighting took place in the fifth round of encounters engaged in by this group of mice. The mice of black Set III (Fig. 1, *b*), which were normal animals, similarly treated, also began attacking and fighting in the second round but exhibited these behavior signs more times than did the mice of black Set VII during this round. However, in Set III, the amount of such aggression decreased from the original high value until, by the fifth round, 15 attacks and 6 fights were displayed, as compared with 59 attacks and 51 fights, which occurred during the fifth round of Set VII.

The behavior of two groups of normal

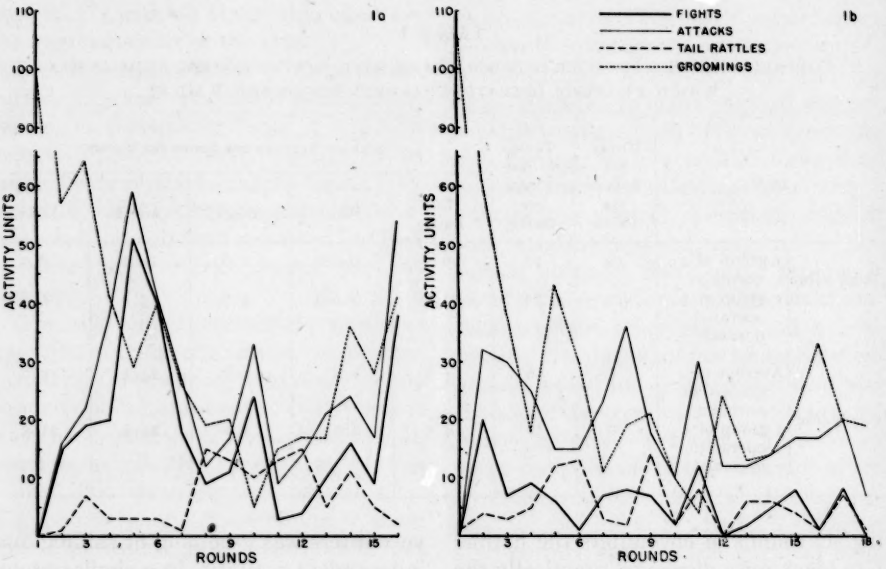


FIG. 1.—Average aggressive activity of normal C57 black male mice in groups of four. *a*, Set VII; *b*, Set III. Note that activity units between 66 and 89 are omitted.

albino mice—Sets V and IX (Fig. 2)—was qualitatively similar to that of the normal C57 black mice with regard to the time of first appearance of components of the aggressive behavior pattern and of occurrence of fluctuations in amounts of these components. As in the blacks, the encounters during the first round were low in aggressive behavior; no aggression of any sort appeared in one group, and only 3 tail rattles were shows that, although the normal mice of Set V were the first to show signs of aggressiveness by displaying 3 tail rattles in the first round, as compared with no aggressive activity on the part of the control-operated mice, the latter group—Set IX—exhibited far more aggressive behavior during the first six rounds than did the unoperated group. During these six rounds the control-operated mice gave 215 tail rattles and engaged in 100

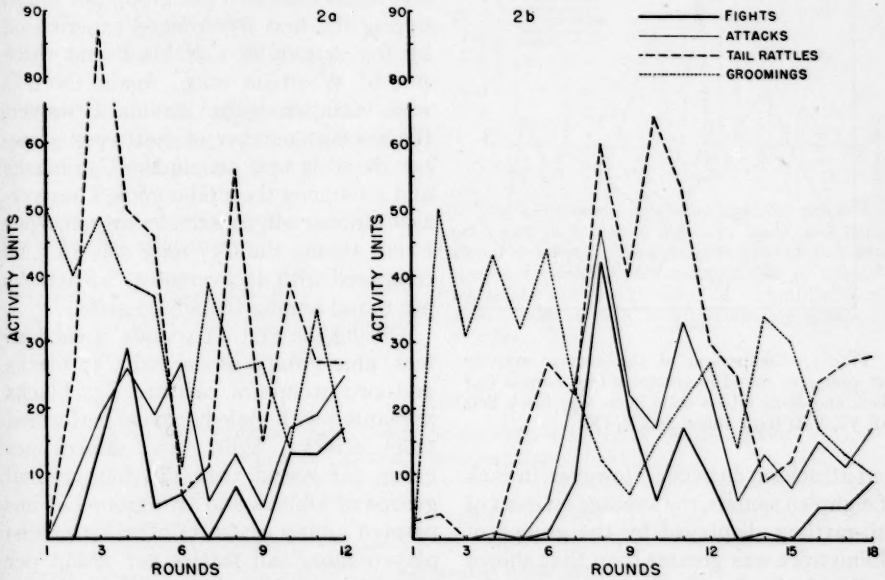


FIG. 2.—Average aggressive activity of normal Bagg albino male mice in groups of four. *a*, Set IX (operated normals); *b*, Set V. Note that activity units between 66 and 79 are omitted.

exhibited by the other. In the second round, however, Set IX displayed 25 tail rattles and 9 attacks. By the third round the aggressive activity of this group had increased to 87 tail rattles, 6 minces, 19 attacks, and 15 fights.

It has previously been indicated that the control operation did not quantitatively depress aggressive behavior and that the operated groups were, indeed, on the average, slightly more aggressive than unoperated groups of mice. Figure 2 attacks and 56 fights, in contrast with 49 tail rattles, 7 attacks, and 1 fight performed by the normal mice in six similar rounds of encounters.

COMPARISON OF THE BEHAVIOR OF NORMAL BLACK MICE WITH THAT OF NORMAL ALBINO MICE

Although the aggressive behavior of the C57 black mice appears to be qualitatively like that of the albino mice, in some respects it is not quantitatively

similar, since there are differences in the frequency with which normal males of these two strains of mice display various signs of aggressive behavior.

The average amount of attacking and tail-rattling per group per round for three groups of normal B C57 black and two groups of normal B albino mice is shown in Figure 3. It is evident that large fluctuations in the amount of tail-rattling

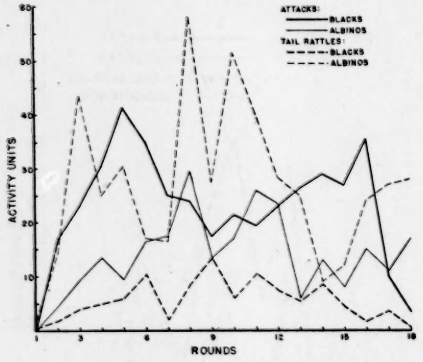


FIG. 3.—Comparison of the average activity per group per round in groups of four normal C57 black and Bagg albino male mice. C57 black Sets III, VI, VII; Bagg albino Sets V, IX.

and attacking do occur. However, in each of eighteen rounds, the average amount of tail-rattling displayed by the groups of albino mice was greater than that shown by the black mice. On the other hand, the albinos exceeded the blacks in the amount of attacking in only five of seventeen rounds. The general trend is for the blacks to exhibit more fighting per round. For example, during eighteen rounds the black mice displayed an average of 11.4 fights per group per round, as compared with an average of 6.3 fights per group per round on the part of the albinos (Table 2). In twelve of the eighteen rounds, the blacks displayed more fighting and exceeded the albinos by an average of 10.9 fights per round. During the five remaining rounds the albinos displayed an average of 8 more fights than did the blacks.

If the aggressive activity of comparable castrated mice of both strains is compared after implantation of testosterone propionate, the albino mice are again much more active in displaying tail-rattling, and, in five out of six rounds, the blacks minced, attacked, and fought more than did the albinos. Figure 4 lists the average number of attacks and fights exhibited per group per round during the first five rounds experienced by five sets of W C57 black and three sets of W albino mice. Again there is wide variation within strains. However, the average number of fights per group per round is 17.2 among the C57 blacks and 2.1 among the albino mice. The average number of attacks per group per round among the C57 mice was 16.5, as compared with an average of 2.8 attacks per round among the albino mice.

During the first six rounds of encounters, nine groups of normal C57 blacks and two groups of castrate C57 blacks implanted with male hormone performed more attacks, fights, and minces per group per round than did four normal groups of albinos and two groups of implanted albino castrates. The latter displayed more tail rattles per group per round than did the former (Table 2).

There is a tendency for the mice to be relatively unaggressive during the first rounds of encounters, to be increasingly aggressive during later encounters, and, finally, after meeting in numerous encounters, to show decreased aggression. In later encounters the mice tend to submit rather than to fight when attacked; and, since encounters were terminated after a submission was given, the amount of aggression exhibited was usually less than that found in many earlier rounds. In addition to variability in performance from round to round within a given group

of mice, the variability between groups of mice of one strain is such that, with the data from the available groups of mice, the difference in any behavior trait between strains is not statistically significant. However, in the first six rounds of encounters, each of eleven groups of C57 black mice displayed more attacks than tail rattles, and each of six groups of albino mice displayed more tail rattles than attacks. When the ratio

$$\frac{\text{Attacks}}{\text{Tail rattles}+\text{attacks}}$$

in both strains is compared, the difference between the two strains is highly significant: $P < 0.0001$ $(t = 11+)$.[3]

[3] The population formula of Fisher was used. Degrees of freedom are $n_1 + n_2 - 2$.

$$t = \frac{\bar{v}_1 - \bar{v}_2}{\sqrt{\left(\frac{(\Sigma v_1^2 - \bar{v}_1 \Sigma v_1) + (\Sigma v_2^2 - \bar{v}_2 \Sigma v_2)}{n_1 + n_2 - 2}\right) \times \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}.$$

EFFECTS OF CASTRATION ON THE AGGRESSIVE BEHAVIOR OF MALE MICE

Practically no aggression was shown in the 198 encounters engaged in by the twenty-three castrate albino mice which were members of six sets (Table 3). The sole castrate of this strain to display any aggressive behavior rattled his tail twice

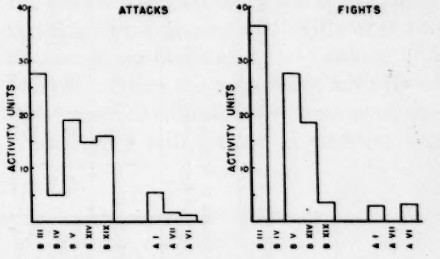


FIG. 4.—Average aggressive activity per group per round in groups of four C57 black and Bagg albino male mice, based on data for the first five rounds of encounters for each group of W mice. *B* represents C57 black males; *A* represents Bagg albino males. Roman numerals indicate groups of four mice.

TABLE 2

DIFFERENCE BETWEEN THE AMOUNT OF AGGRESSIVE ACTIVITY PER GROUP PER ROUND OF C57 BLACK AND BAGG ALBINO MICE

		Attack	Fight	Mince	Tail Rattle
	Normal Groups of 4 Mice (Rounds 1–18)				
B mice.....	3 groups of C57 blacks	20.4	11.4	4.6	5.5
	2 groups of albinos	13.9	6.3	3.6	26.6
	Castrate Groups of 4 Mice with Pellets of Testosterone Propionate (Rounds 7–12)				
B mice.....	2 groups of C57 blacks	25.6	18.0	10.3	12.4
	2 groups of albinos	16.3	10.8	3.8	37.8
	Groups of 4 Mice, Normal, or Castrate with Pellets of Testosterone Propionate (Rounds 1–6)				
B and W mice..	11 groups of C57 blacks	19.8	11.9	3.5	5.1
	6 groups of albinos	9.3	5.7	2.4	22.2

TABLE 3

AGGRESSIVE BEHAVIOR OF MICE: NORMALS, CASTRATES, AND CASTRATES IMPLANTED WITH TESTOSTERONE PROPIONATE OR DEXTROSE PELLETS

Strain	Set	No. Mice per Set	Activity in First 6 Rounds of Encounters					Treatment	Activity in Second 6 Rounds of Encounters					Treatment	Activity in Third 6 Rounds of Encounters				
			Tail Rattle	Parry	Mince	Attack	Fight		Tail Rattle	Parry	Mince	Attack	Fight		Tail Rattle	Parry	Mince	Attack	Fight
Albino ♂	I	3	0	0	0	0	0	T.P.*	138	1	26	79	58						
Albino ♂	II	4	0	0	0	0	0	T.P.	267	1	24	117	104	T.P. out	1	0	1	0	0
Albino ♂	VI	4	2	0	0	0	0	T.P.	187	0	23	79	26						
Albino ♂	III	4	0	0	0	0	0	Dextrose	0	0	0	0	0		0	0	0	0	0
Albino ♂	VIII	4	0	0	0	0	0	Dextrose	0	0	0	0	0						
Albino ♂	VII	4	0	0	0	0	0												
Normal ♂	V	4	49	0	1	7	1		269	1	18	169	102		125	0	28	70	20
Normal ♂ operated	IX	4	215	0	23	100	56		175	1	34	85	51						
C57 black ♂	II	3	2	0	0	0	0	T.P.	27	8	29	48	18	T.P. out	3	0	0	3	0
C57 black ♂	IX	3	0	0	0	0	0	T.P.	1	0	15	35	0						
C57 black ♂	I	4	0	0	0	0	0	T.P.	59	13	86	150	87	T.P. out	22	0	26	47	0†
C57 black ♂	V	4	0	0	0	0	0	T.P.	90	14	38	158	129						
C57 black ♂	IV	3	0	0	0	0	0	Dextrose	0	0	0	0	0						
C57 black ♂	VIII	4	0	0	0	0	0	Dextrose	0	0	0	0	0						
C57 black ♂	XI	4	0	0	0	0	0												
Normal ♂	III	4	38	8	11	117	43		29	9	37	129	36		25	7	30	87	24
Normal ♂	VII	4	17	13	11	176	145		67	6	38	112	69						
Normal ♂ operated	VI	4	27	12	49	147	129												

* Testosterone propionate.

† All aggressive behavior in third six rounds was displayed by Mouse No. 2, whose androgen pellet was removed in three pieces.

in the 17th encounter, which occurred during the 6th round of Set VI. He was one of a group of four mice which had been castrated at 80 days and had given no indication of aggressiveness in the sixteen preceding encounters. The aggressive behavior of the twenty-five castrate C57 black mice (seven sets), in the same number of encounters, also consisted of only 2 tail rattles (Table 3). These were displayed during the first round of encounters engaged in by Set II, which had been castrated at 23 days. In the total of 396 encounters experienced by castrate mice of both strains, none of the forty-eight mice showed any other sign of aggressiveness.

It is obvious that the removal of the gonads from male Bagg albino and C57 black mice results in the failure of the castrated mice to act aggressively, when a period of 25 or more days intervenes between castration and initial encounters. This is true if the mice are castrated as immature males or if castration occurs after 60 days of age, when they are sexually mature. In addition, the age at which they were previously isolated apparently has no effect on their behavior, and the number of encounters experienced does not appear to be of importance in inducing aggressive behavior in castrate mice, since the thirteen sets of castrate mice were, in all cases, permitted to meet in a minimum of six rounds of encounters; four sets experienced twelve rounds; and one set was run for eighteen rounds. It has been stated that male mice of both strains normally exhibit large amounts of various types of aggressive behavior at least by the second round of encounters.

This paucity of aggressive activity on the part of the castrate mice is especially striking when compared with the behavior of normal animals (Table 3). In 180 encounters the aggressive activity of eight normal albino mice (two sets) included 833 tail rattles, 2 parries, 104 minces, 431 attacks, and 230 fights. Twelve normal C57 black mice (three sets), in 240 encounters, rattled their tails 226 times, exhibited 69 parries, 180 minces, 884 attacks, and 518 fights. Since the total number of encounters in which the groups of castrates and groups of normal mice engaged was not the same, these results have been compared in Table 4 in terms of the average activity exhibited per encounter.

TABLE 4

COMPARISON OF THE AVERAGE ACTIVITY PER ENCOUNTER IN GROUPS OF NORMAL AND CASTRATE C57 BLACK AND ALBINO MICE

Group	Total No. of Encounters	Average Activity per Encounter				
		Tail Rattle	Parry	Mince	Attack	Fight
23 castrate albino mice (6 groups)	198	0.010	0	0	0	0
25 castrate black mice (7 groups)	198	0.010	0	0	0	0
8 normal albino mice (2 groups)	180	4.6	0.011	0.57	2.3	1.2
12 normal black mice (3 groups)	240	0.94	0.24	0.75	3.6	2.1

Castrate mice, as a group, displayed more grooming or sniffing than did the normal mice. As indicated in Table 5, the castrate albinos averaged 7.3 groomings per encounter, as contrasted with 4.47 per encounter displayed by normal albino males. The difference between the behavior of castrate and normal C57

blacks is even greater; 14.9 groomings were engaged in per encounter by the castrates, as compared with 5.48 per encounter by the normals.

Squeaking, submitting, and running away are signs of submissive behavior. It is not surprising that the normal mice of both strains displayed these acts more frequently in each encounter than did the castrates of the same strain, since the former were actively engaging in attacks and fights and mice normally submit when beaten. It is of interest that the castrate mice of both strains showed any squeaking, submitting, and running away from other mice in the absence of active aggressive behavior, especially since Ginsburg and Allee (1942) state: "Submissive responses to aggressiveness do not usually occur until after several defeats, and this is an indication that learning is an important factor in such submissions" (p. 501). The twenty-three castrate albino mice squeaked once, submitted once, and ran away three times in 198 encounters. In the same number of encounters, the C57 black castrates showed much more submissive behavior with 37 submissions, 6 squeaks, and 20 instances of flight.

TABLE 5

SUBMISSIVE BEHAVIOR AND GROOMING IN NORMAL AND CASTRATE C57 BLACK AND ALBINO MICE

GROUP	NO. OF ENCOUNTERS	AVERAGE ACTIVITY PER ENCOUNTER			
		Groom	Squeak	Submit	Run Away
6 sets albino ♂	198	7.3	0.005	0.005	0.015
7 sets C57 ♂	198	14.9	0.030	0.186	0.10
2 sets albino ♂	180	4.47	1.35	0.57	0.57
3 sets C57 ♂	240	5.48	1.3	0.72	1.0

EFFECTS OF CASTRATION ON BODY WEIGHT

In all cases the average weights of the groups of castrate mice were consistently less than those of groups of normal mice of comparable strain and age. The average body weights among the four groups of castrate albinos ranged from 4.2 to 6.9 gm. less than the average weights of two groups of normal albino mice of comparable ages. The average body weights of six groups of castrate C57 blacks ranged from 1.3 to 6.2 gm. less than the average weights of three groups of normal mice of the same strain and age.

IMPLANTATION OF PELLETS OF TESTOSTERONE PROPIONATE IN CASTRATE C57 BLACK MICE

A dramatic change occurred in the behavior of the members of four groups of castrate C57 black mice soon after the implantation of pellets of testosterone propionate. The pellets were introduced after each group had completed six rounds of encounters, and they were not removed until 3 weeks later, when six additional rounds of encounters had occurred.

The seventh round for Set I, a group of four mice, was run on the next day after the pellets were implanted. On this day, one of the mice started to attack another mouse but stopped before carrying the act to completion. During the 8th round, which occurred 4 days after the implantation of the pellets, this group displayed 5 tail rattles, 7 minces, 47 attacks, 5 parries, and 27 fights—behavior

never exhibited by any of the four groups of castrate black mice in six previous rounds (Fig. 5, *a*). Three of the four mice in the group continued to be extremely aggressive during the entire period of time that the pellets of androgen were present. In the 12th round (6th round after introduction of pellets) these mice gave 20 tail rattles, 17 minces, 16 attacks, and 7 fights. Mouse No. 4, however, failed to exhibit any sign of aggressiveness.

The mice were allowed to experience a third set of six rounds of encounters after the pellets had been removed. During rounds thirteen through eighteen, mice Nos. 1, 3, and 4 did not perform a single aggressive act. Mouse No. 2, however, continued to rattle his tail and to mince for four more rounds and to attack during all the last six rounds. There were no fights, since the other mice merely submitted and squeaked or ran away when No. 2 acted aggressively. The pellet of this mouse was removed in three pieces, and the weight of these pieces suggests that the entire pellet was not retrieved.

The 7th round of encounters for Set V occurred 4 days after implantation of testosterone propionate pellets. At this time these mice gave 6 minces, 18 attacks, 9 parries, and 10 fights. The change in behavior which developed in all four mice during the rounds when the pellets of androgen were present are illustrated in Figure 5, *b*. The graph indicates that, although the amount of aggressive activity exhibited in any one round varied from that displayed in subsequent rounds, the mice continued to show signs of aggressiveness during the entire second group of encounters, rounds seven through twelve. Figure 5, *b*, also shows the drop in grooming which occurred when aggressive activity increased and the rise in grooming which took place when the amount of aggressive activity decreased.

The members of a third group of C57 black castrates, Set II, experienced the 7th round of encounters 2 days after receiving male hormone in subcutaneously implanted pellets. No change occurred in the behavior of this group of three mice during this round. They continued to spend time in grooming and independent activity. During the 8th round, which was staged on the 6th day after implantation, the three mice gave a total of 2 tail rattles, 10 minces, 11 attacks, 2 parries, and 6 fights (Fig. 5, *c*). All the mice continued to be aggressive in the 9th, 10th, 11th, and 12th rounds. In the six rounds after the pellets had been removed, mice Nos. 2 and 3 failed to show any signs of aggressiveness. Mouse No. 1 made 3 attacks, 1 in the 13th round and 2 in the 17th round. He also gave 3 tail rattles, 1 in the 17th and 2 in the 18th round.

The behavior of a fourth group of castrate C57 mice—Set IX—differed from that described for the three other groups of castrate mice which received pellets of male hormone. Unlike the members of Sets I, II, and V, the mice in this group never fought. In addition, the frequency with which tail-rattling, mincing, and attacking appeared was lower than that found in the other three sets. No aggressive behavior signs were displayed during the first round after implantation of the pellets. However, some aggression by No. 107 appeared in each succeeding round until the experiment was terminated after the 12th round. Mouse No. 108 never displayed any aggressiveness, and No. 109 displayed only 1 attack during the 12th round. The pellets of Nos. 108 and 109 were not recovered at autopsy, and it is possible that they were lost soon after implantation. The weights of the seminal vesicles and prostate glands lend support to this hypothesis.

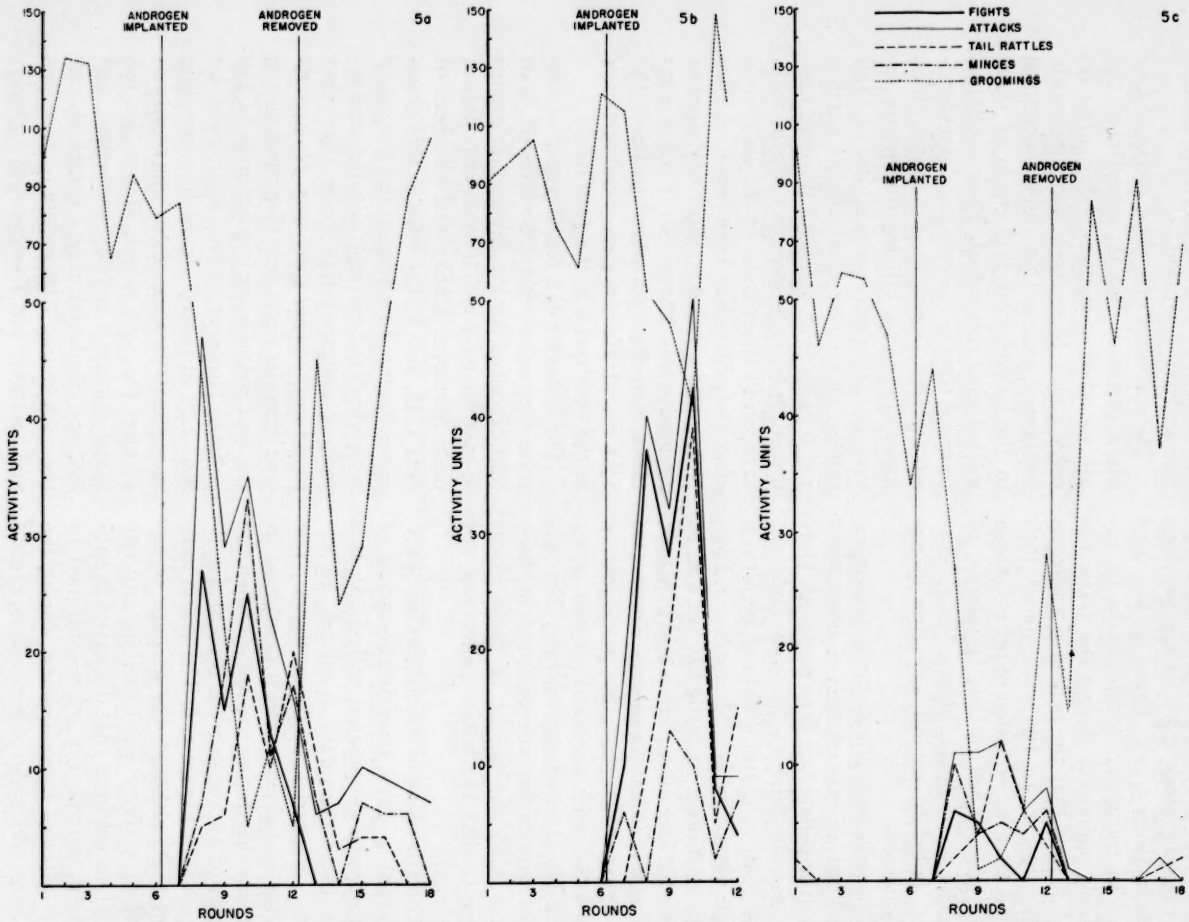


FIG. 5. Average aggressive activity of two groups of four and one group of three castrate C57 black male mice. Pellets of testosterone propionate present in rounds seven through twelve. *a*, Set I, group of 4 males. All aggressive behavior after round twelve was displayed by mouse No. 2, whose pellet was removed in three pieces (note that fighting ceases after round twelve). *b*, Set V, group of 4 males. *c*, Set II, group of 3 males. Note that the scale for activity units is reduced between 50 and 150.

In summary, twelve out of fourteen castrate C57 black mice which had received pellets of male hormone exhibited aggressive behavior. Aggressive activity was first displayed in encounters run on the 4th to the 6th day after implantation of androgen pellets, and the mice continued to show some signs of aggressiveness during subsequent encounters. Seven of these mice experienced encounters following the removal of the pellets, and only two of these showed any aggression.

IMPLANTATION OF PELLETS OF TESTOSTERONE PROPIONATE IN CASTRATE BAGG ALBINO MICE

After the castrate albino males of Sets I, II, and VI had completed six rounds of encounters, they received pellets of testosterone propionate. All were allowed to experience six additional rounds of encounters while implanted with male hormone, and the members of Set II were given a third set of six rounds after removal of the pellets.

In Set II the first round of encounters occurred 4 days after the pellets were implanted. All the mice in the group were actively aggressive at this time. During this round these previously unaggressive mice displayed 34 tail rattles, 2 minces, 28 attacks, and 29 fights. As indicated in Figure 6, *a*, the members of Set II continued to be aggressive throughout the six rounds of encounters which took place while the mice were carrying the pellets of androgen. Pellets were removed from three of the four mice of Set II after the 12th round. Since the pellet of No. 2 could not be found, only mice Nos. 1, 3, and 4 were permitted to continue in a third six rounds of encounters. Figure 6, *a*, clearly indicates that the aggressive behavior disappeared upon removal of the androgen pellets except for 1 tail rattle and 1 mince, which occurred in the first round after withdrawal of the hormone. As in the case of the C57 black mice, the amount of grooming and sniffing decreased when the aggressive behavior began and rose sharply in amount when the pellets of male hormone were removed.

The mice in albino Set VI (Fig. 6, *b*) first exhibited aggression in the 7th round, 4 days after having received pellets of testosterone propionate. Only two mice—Nos. 106 and 131—displayed signs of aggressiveness at this time. During the 8th round, mouse No. 129 also began to act aggressively, and it was not until the 9th round that the fourth member of Set VI—No. 130—showed the characteristic aggressive behavior pattern. Aggressive signs were displayed throughout the next three rounds until the end of the experiment.

The 7th round for members of Set I occurred 2 days after the pellets had been implanted, but no change in the behavior of the mice was seen. It was not until 4 days later, in the 8th round, that all the mice were actively aggressive (Fig. 6, *c*). This behavior continued in the 9th, 10th, 11th, and 12th rounds, with a total of 138 tail rattles, 1 parry, 26 minces, 79 attacks, and 58 fights occurring before the encounters were ended at the close of the 12th round.

In summary, the eleven albinos, members of three castrate groups, received subcutaneous pellets of testosterone propionate, and all showed aggressive activity at sometime during the six rounds of encounters when male hormone was present. Table 3 includes the total amount of aggressive behavior displayed by the three groups of albino and four groups of C57 black castrates during the period in which they possessed pellets of testosterone propionate and in the periods preceding and following implantation.

It is interesting to compare the ac-

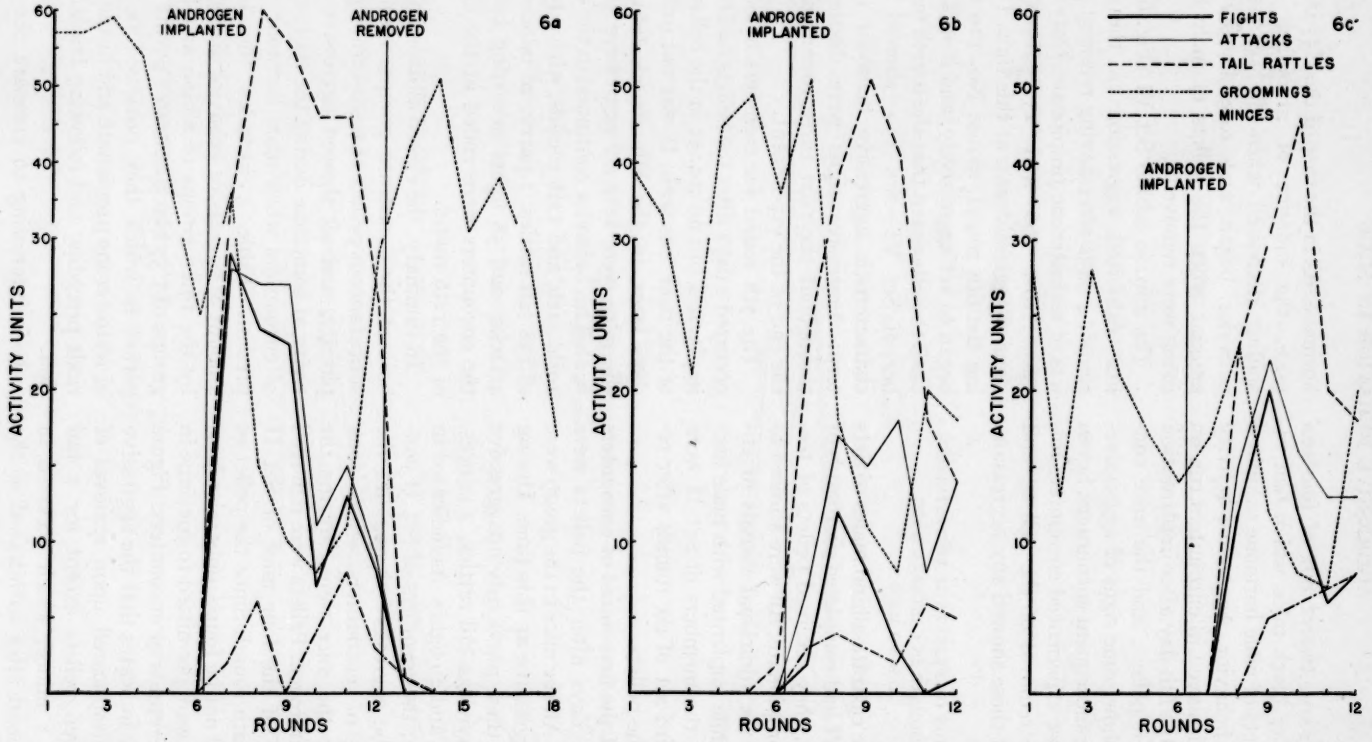


FIG. 6.—Average aggressive activity of two groups of four and one group of three castrate Bagg albino male mice. Pellets of testosterone propionate present in rounds seven through twelve. *a*, Set II, group of 4 males. Only three mice present in rounds thirteen to eighteen. *b*, Set VI, group of 4 males. *c*, Set I, group of 3 males. Note that the scale for activity units is reduced between 30 and 60.

tivity of the castrate mice under the influence of androgen pellets with that of normal mice of the same strain. Such comparisons are made in Table 6. Although the first encounters during which the castrate mice with implanted male hormone were aggressive actually occurred during the second group of six rounds, from the point of view of past experience in aggressive behavior, these encounters are comparable to the first six rounds of encounters experienced by normal mice.

The castrate C57 black mice exhibited more tail-rattling, parrying, mincing, attacking, and fighting during the six rounds of encounters when pellets of testosterone propionate were present than did normal C57 black mice during the first or second group of six rounds of encounters.

The castrate albino mice with testosterone propionate pellets exhibited more of typically aggressive behavior than did the normal albinos in the first six rounds of encounters. During the second six rounds of encounters, however, these normal mice displayed slightly more mincing, attacking, and fighting than did the castrate mice possessing implanted male hormone.

In both strains of mice an increase in one or more types of aggressive activity occurred in the rounds immediately following the initial aggressiveness induced by the androgen. A peak of aggressive behavior was observed in the 2d, 3d, or 4th rounds, following the implantation of male hormone in each of the seven groups of castrates so treated; and this rise was followed, in most cases, by a decrease in the amount of all types of aggressiveness displayed (Figs. 5 and 6). The decrease began in the 9th or 10th round among the albinos and in the 11th round in two groups of black mice. After this drop in total aggressive activity, a slight rise in one or more types of activity took place in five of the seven groups of treated castrates.

The above trends in aggressiveness may be compared with similar peaks and decreases of activity which may occur in groups of normal C57 black and albino mice. Black Set VII (Fig. 1, *a*) and albino Set IX (Fig. 2, *a*) show sharp increases in aggressive behavior following initial encounters, with subsequent decreases in activity. Although the mice in albino Set V were slow to begin aggressive activity, they also showed similar increases and decreases in aggression.

TABLE 6

AVERAGE AGGRESSIVE ACTIVITY OF GROUPS OF FOUR MICE DURING PERIODS OF SIX ROUNDS
(Each category is based on totals from two groups of mice)

Group	Tail Rattle	Parry	Mince	Attack	Fight
Albino ♂ with T.P.	227.0	0.5	23.5	98.0	65.0
Albino normals (1st 6 rounds)	132.0	0	12.0	53.5	48.5
Albino normals (2d 6 rounds)	222.0	1.0	26.0	127.0	76.5
Black ♂ with T.P.	74.5	13.5	62.0	154.0	108.0
Black normals (1st 6 rounds)	27.5	10.5	11.0	146.5	94.0
Black normals (2d 6 rounds)	48.0	7.5	37.5	120.5	52.5

AGGRESSIVE ACTIVITY OF ANDROGEN-IMPLANTED CASTRATE MICE IN GROUPS OF THREE AND IN GROUPS OF FOUR

There seems to be a difference between the amount of aggressive activity displayed by individual mice in groups of three C57 black castrate mice with im-

planted pellets of testosterone propionate and that exhibited by similar mice in groups of four. Since data are available on only two groups of mice in each of these categories, statistical treatment is not possible. However, as indicated in Figure 7, any two mice which were members of groups of four mice performed more tail-rattling, attacking, and fighting in a single encounter during each of six rounds than did mice which were members of groups of three. At present no ex-

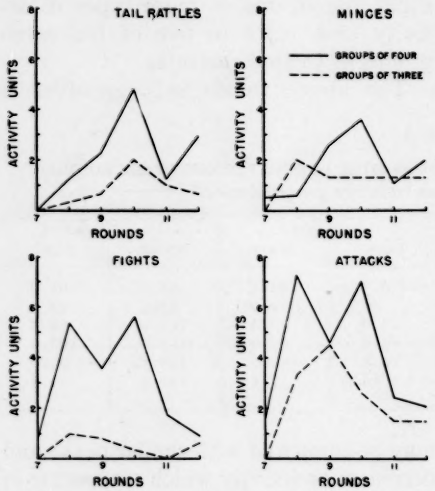


FIG. 7.—Comparison of the average amount of aggressive activity per encounter per round in androgen-implanted castrate C57 black male mice in groups of three with that in groups of four. Groups of three, Sets II, IX; groups of four, Sets I, V.

planation can be offered to account for the stimulating effect which an additional encounter apparently had on the total aggressiveness shown in other encounters.

EFFECT OF CASTRATION AND IMPLANTATION OF TESTOSTERONE PROPIONATE ON THE WEIGHTS OF SEMINAL VESICLES AND PROSTATE GLANDS

It has been known for many years that the size and function of the seminal vesicles and prostate glands are controlled by testicular hormone and that these glands are reduced in size in animals that have been castrated for some time (reviewed in Moore, 1939). It has been demonstrated that the seminal vesicles and prostates of the mouse atrophy rapidly after castration. Martins and Rocha E Silva (1929) have reported that within 10–13 days following gonadectomy there is a marked reduction in the size of the seminal vesicles. However, since they found that the weight reduction was variable in mice of over 17–18 gm., they utilized length-times-breadth measurements of these glands as an indication of the amount of androgen present. Deanesly and Parkes (1933) have found that within 7 days after postpubertal castration in mice, about half the combined weight of the seminal vesicles and anterior prostatic lobes is lost. By 3 weeks after castration, the atrophy is considerably advanced, and further slight decreases in weight follow up to 7 weeks. A nearly constant level of less than 10 per cent of the original glandular weight is gradually attained. These authors disagree with Martins and Rocha E Silva (1929), believing that there is less variation in weight in the glands of mice over 24 gm. than in those of smaller mice.

Confirmation of the dependence of seminal vesicle and prostate size on male hormone has been attained in the present study. Since it has been shown that the aggressive behavior of mice also is affected by the presence of testicular hormone, it is to be anticipated that a correlation may be found between the size of these male accessory glands and the aggressive activity of these animals.

The mice of C57 black Sets IX and V and albino Sets I and VI, which contained androgen pellets during the 6th through the 12th rounds of encounters, were killed, and the pellets were removed

immediately after the end of the 12th round. Pellets of male hormone also were removed from the members of C57 black Sets I and II, and albino Set II at the end of the 12th round after these mice had been carrying the pellets for six rounds, a period of 3 weeks. However, the members of these three sets experienced a third set of six rounds of enof the same strain was 7.0 mg. In albino mice the average weight of the glands of six normal mice was 195.71 mg., and 6.18 mg. for the glands of eight castrates. There was no significant difference between the average weight of the seminal vesicles of normal mice of both strains and that of the castrate mice of these strains in which the pellets of androgen

TABLE 7

EFFECT OF CASTRATION, IMPLANTATION, AND REMOVAL OF PELLETS OF TESTOSTERONE PROPIONATE ON THE WEIGHT OF SEMINAL VESICLES AND PROSTATE GLANDS OF C57 BLACK AND BAGG ALBINO MICE

Difference between the Weight of Organs of:	*P*-Values and Average Weight of Glands*			
	C57 Black Mice		Albino Mice	
	Seminal Vesicles	Prostates	Seminal Vesicles	Prostates
Castrates	<0.0002†	<0.0002†	<0.0002†	<0.0002†
	7.0	1.9	6.2	2.95
Normals	264.0	17.8	195.7	20.7
Androgen out at autopsy	0.058	>0.5	>0.5	<0.0002‡
	153.3	15.5	188.8	25.4
Normals	264.0	17.8	195.7	20.7
Androgen out at autopsy	0.0095†	0.0075†	<0.0002†	<0.0002†
	153.3	15.5	188.8	25.4
Castrates	7.0	1.9	6.2	2.95
Androgen out at autopsy	>0.5	0.0214	<0.0002	<0.0002
	153.3	15.5	188.8	25.4
Androgen out 21–22 days before autopsy	129.0	4.2	15.8	4.03
Androgen out 21–22 days before autopsy	0.01†	0.0030†	<0.0002†	0.12
	129.0	4.2	15.8	4.03
Castrates	7.0	1.9	6.2	2.95

* *P*-values are given in the first line. Average weights of glands in milligrams are given in the second and third lines.
† Larger than castrates. ‡ Larger than normals.

counters and were killed 21–22 days after removal of the pellets (Table 3).

Seminal vesicles.—The seminal vesicles of the castrate C57 black and albino mice weighed significantly less than these glands in normal mice of both strains, i.e., $P < 0.0002$ in both cases (Table 7). The average weight of the seminal vesicles in eleven normal C57 blacks was 264.0 mg.; that of seven castrate mice were removed on the day of autopsy. Since the accessory glands of the castrate C57 black and albino mice from which pellets of male hormone were removed at autopsy were as large as, or larger than, the seminal vesicles of normal mice, it is not surprising that these glands were also significantly larger than those of castrate mice of the same strain.

The seminal vesicles of castrate C57

black mice whose pellets were removed on the day of autopsy averaged 153.3 mg.; those of castrate black mice from which the pellets were removed 21–22 days before autopsy averaged 129.0 mg. There is no significant difference between these weights, indicating that the seminal vesicles of the black mice did not regress in weight rapidly after the withdrawal of male hormone. The C57 black mice from which androgen pellets were removed 22 days before autopsy averaged 80.0 days of age when castrated. Those from which the pellets were removed on the day of autopsy averaged 42.4 days of age at castration. According to Martins and Rocha E Silva (1929), in older animals the atrophy is irregular and sometimes retarded; and Kochakian (1941) states that animals which are older at time of castration exhibit a greater response to comparable amounts of androgen. The difference between the seminal vesicles of albino mice in these two categories is highly significant ($P < 0.0002$). These facts may explain the "apparent" slow regression in weight of the seminal vesicles in the former group after pellet removal. There was a difference of only 13 days between the ages of these two groups of albinos. Thus, 21–22 days after removal of pellets of male hormone, the seminal vesicles of the albino castrates had significantly decreased in size.

Since it has already been shown that 21–22 days after removal of androgen pellets the seminal vesicles of the C57 mice are approximately the same size as those of the mice which had the pellets removed on the day of autopsy and since the difference between the weights of the seminal vesicles of the latter mice and normals is insignificant, it is to be expected that the weights of these glands in mice whose pellets had been removed 21–22 days before autopsy would be significantly greater than those of the seminal vesicles of castrate C57 black mice, e.g., P is found to be 0.01.

Prostate glands.—The prostate glands of castrate mice of both strains weighed significantly less than these glands did in comparable normal mice. The average weight of the prostate glands of twelve normal black mice and seven castrate black mice was 17.8 and 1.85 mg., respectively. The prostates of seven normal albino mice averaged 20.7 mg. as compared with 2.95 mg. for eight castrate albino mice.

There was no significant difference between the average weight of the prostate glands of normal C57 black mice and that of castrate black mice from which the pellets of male hormone had been removed at autopsy. However, the difference between the prostate gland weights of albino mice in these two categories is significant; the normal glands are much larger.

The mice of both strains from which the pellets of testosterone propionate had been removed on the day of autopsy possessed prostate glands which were significantly larger than were these glands in castrate mice of the same strain.

The prostate glands of six C57 black castrates which had had the pellets removed on the day of autopsy averaged 15.5 mg., in contrast with an average of 4.2 mg. for the prostate glands of five C57 black castrates which had been deprived of pellets for 21–22 days at autopsy. This difference in weight is significant: $P = 0.021$. Thus the prostate glands of the castrate C57 black mice regress more rapidly after the withdrawal of male hormone than do the seminal vesicles of these mice. The difference between the average weight of the prostate glands of the albino mice in these two categories is also significant. Thus, 21–22 days after removal of pellets of male hormone, the prostate glands of the albino

castrates have also decreased significantly in size.

The prostates of the C57 castrate mice deprived of androgen for 21–22 days before autopsy were also significantly larger than those of castrates, indicating that, although they weighed significantly less than those of C57 mice whose pellets were removed at autopsy, they had not yet regressed to the castrate level. However, 21–22 days after removal of pellets of male hormone, the average weights of these glands in castrate albino mice had reached a size which was not significantly larger than that of castrate mice which had never had pellets implanted.

The P-values[4] listed above have been calculated on the data from small numbers of mice, since only data from those mice from which both seminal vesicles or both prostate glands had been removed intact were used. In the mice which had been castrated for some time it was difficult to find and completely remove the very small glands.

The amount of aggressive behavior displayed by normal and castrate mice and by castrate mice from which androgen pellets had been removed at autopsy or 21–22 days before autopsy is compared in Table 8 with the size of the seminal vesicles and prostate glands of these animals. It is evident that the amount of aggression displayed is fairly closely correlated with the size of these male accessory glands. When the glands are approximately the same weight in two

TABLE 8

CORRELATION OF THE EFFECTS OF CASTRATION AND IMPLANTATION OF TESTOSTERONE PROPIONATE ON THE SIZE OF SEMINAL VESICLES AND PROSTATE GLANDS OF C57 BLACK AND BAGG ALBINO MICE WITH THE AMOUNT OF AGGRESSIVE BEHAVIOR DISPLAYED BY THESE MICE

Groups of Mice	Strain	Weight of Glands	Amount of Aggressive Behavior
Castrates with androgen pellets removed at autopsy	Blacks and albinos	Same as normals*	Same as normals
	Blacks and albinos	Heavier than castrates*	Greater than castrates
	Blacks	Seminal vesicles same as those in mice with androgen removed 21–22 days before autopsy Prostates heavier than those in mice with androgen removed 21–22 days before autopsy	With 1 exception, greater than those mice with androgen removed 21–22 days before autopsy
	Albinos	Heavier than those in mice with androgen removed 21–22 days before autopsy*	Greater than those mice with androgen removed 21–22 days before autopsy
Normal mice	Blacks and albinos	Heavier than castrates*	Greater than castrates
Castrates with androgen pellets removed 21–22 days prior to autopsy	Blacks	Heavier than castrates*	Greater than castrates (1 exception)
	Albinos	Seminal vesicles heavier than castrates Prostates same as castrates	Same as castrates

* Both seminal vesicles and prostates.

[4] $t = \dfrac{\bar{x}}{\sqrt{\dfrac{\Sigma x^2 - \bar{x}\Sigma x}{n(n-1)}}}$; degrees of freedom $= n-1$.

groups of mice, the behavior is also approximately the same. Or, if the glands are larger in one group than in the other, the former displays more aggression than the latter. This correlation results, of course, from the fact that both the size of the accessory glands and the amount of aggressive behavior displayed are affected by the amount of male hormone which is present.

EFFECTS OF PELLETS OF DEXTROSE

No change occurred in the behavior of the members of two sets of albino castrates, III and VIII, or in that of the members of two sets of C57 black castrates, IV and VIII, when pellets of dextrose were implanted after each set had had six rounds of encounters. Table 3 indicates that these mice did not display a single aggressive act before the pellets of dextrose were introduced and that absolutely no aggressiveness was apparent in any of the 126 encounters in the second group of six rounds after the pellets had been implanted. Albino Set III was permitted to experience a third group of six rounds of encounters, and all the mice continued to be unaggressive.

DISCUSSION

The present study on aggressiveness has been concerned with normal behavior directed toward other animals of the same species and strain. Under these conditions the males of both the C57 black and the Bagg albino strains exhibit aggressive behavior that is qualitatively similar. There is a general trend for more attacking and fighting on the part of the black mice, and the albinos rattle their tails significantly more than do the C57 blacks. The data previously reported on aggression in these strains have dealt with aggression between strains, primarily with ability to initiate or win encounters and not with the aggressive behavior pattern within a given strain.

Under the particular experimental conditions which were used by Ginsburg and Allee (1942) the C57 blacks were superior, the C3H agoutis were intermediate, and the albinos were least effective in winning battles. From observations based on prefighting and fighting behavior of these strains, Scott (1942) rated the C57 blacks as being pacific under certain conditions, the C3H as exhibiting a moderate degree of activity and a tendency to avoid close contacts, and the albinos as possessing moderate activity and aggressiveness. Results of experiments which present some possible explanations for the difference between the results obtained by Ginsburg and Allee and those reported by Scott will be published later.

It has been shown that the amount of any of the components of the aggressive behavior pattern exhibited in a single 5-minute encounter or in a round of such encounters may differ markedly from the amount displayed in another similar encounter or round. These fluctuations in amount of particular types of behavior in groups of normal males may result from several factors. One of these is concerned with the order in which the mice happen to meet on any given day. If the encounters that an aggressive mouse has at the beginning of a round are with fairly submissive mice, the former may rattle his tail vigorously, mince, and attack repeatedly in subsequent encounters. If, however, the first encounter happens to be between two very aggressive mice, they may attack and fight each other so vigorously that each becomes conditioned to show little aggression in subsequent encounters on that particular day.

Conditioning which extends over a period of time longer than a day may

also influence the amount of aggressive behavior which is displayed. If a mouse has been severely beaten in a series of encounters, he may remain submissive or inactive for a week or more. Recorded aggressive activity may be greatly reduced in a group in which only one mouse is very actively aggressive, since, if his opponents submit at the first sign of aggressive behavior, encounters automatically end. If, on the other hand, two moderately aggressive mice are paired, a single encounter may continue for 5 minutes, with first one mouse attacking or rattling his tail or otherwise exhibiting aggressive behavior and then the other mouse showing signs of taking the initiative. In such an encounter a large amount of aggressive activity would be recorded, with neither mouse necessarily losing or winning the encounter.

Seward (1945*b*) describes behavior of the rat in which decreases and increases in aggressive activity occur which are somewhat similar to those which appear so clearly in various groups of normal and androgen-implanted mice of both strains. He finds a general decline in fighting in the course of a series of contacts, but the rats used recovered their pugnacity in the intervals between series. Apparently, the large amount of aggressive behavior displayed by the mice during the first several rounds of encounters is sufficient to condition them gradually to show less aggression as a group. Subsequent encounters with unaggressive individuals aid in restoring former aggressiveness.

In a later study Seward (1946) reports similar conclusions for behavior of rats, stating:

> The day after a fight the average loser made fewer aggressions or advances than before against the winner and against another rat. Exposure to an inoffensive rat completely restored the average loser's aggressions or advances against that rat, against the winner, and against another animal. One session was sometimes enough, but even after a week of daily sessions the restored aggressiveness collapsed readily [p. 75].

Aggressive behavior in rats has been the subject of various investigations, and in several of these, as in the present experiments, the aggression has been directed against other members of the same hereditary strain. It was found by Davis (1933) that isolated rats which were fed once every 24 hours did not act in an aggressive fashion when strange rats were introduced. If, however, the rats were fed only once every 48 hours, the isolated rats would allow the introduced rats to eat and then would become aggressive, pushing and shoving the strange rat. These results are of interest, in view of the fact that Ginsburg and Allee (1942) found that a 36-hour period of starvation made no difference in the aggressiveness of their mice; they observed only one fight over food, and that did not occur during or after a period of starvation.

A frustrating food situation has been used by Billingslea (1941) to provoke aggressiveness in female rats. When female rats, regularly caged in groups of three, were tested in a food situation that allowed only one rat to eat at a time, "emotional" rats tended to be less aggressive than those from a nonemotional strain. Aggression was measured by recording the number of fights participated in on 6 possible days.

Davis (1933) devised a scale for rating aggressiveness directed against other rats. Hall and Klein (1942) used Davis' scale and, in addition, developed a modification that they believed to be more discriminating. They report that rats selectively bred for fearlessness in a strange situation were more aggressive than those bred for timidity.

Much of the research in this field which has been reported for rodents has dealt either with aggressive behavior di-

rected against the investigator or with tameness and wildness, where the degree of wildness has been indicated by speed in going through a runway or maze. Few data have been given which correlate such "aggressiveness" or "wildness" with aggressive behavior of the type under consideration here. However, these studies do have a bearing on the present problem, since they indicate that factors such as "wildness," "savageness," and "tameness" have a hereditary basis in several strains of rats and mice, as do components of the aggressive behavior pattern which have been the subject of this investigation.

Wildness and tameness, as measured by the length of time required for a given mouse to traverse the length of a 22-foot runway, appear to be inherited characteristics (Dawson, 1932). Wild mice bred from individuals caught in fields ran the distance in a much shorter time than did three strains of tame laboratory mice. The genes responsible for tameness apparently are almost completely recessive to those producing wildness.

Coburn (1922), using a rating scale originally devised by Yerkes (1913) to measure savageness, wildness, and timidity in rats, tested wild mice and tame mice for savageness and wildness. Tests of mice from matings between wild and tame stocks were also made. The aggressive behavior was not directed against other mice but against the investigator. Yerkes' rating scale (1913) for savageness included (1) biting, (2) exposing or gnashing teeth, (3) jumping at hand or forceps, and (4) squeaking. Wildness involved (1) attempts to hide from view in cage or hand, (2) random and excited running about in the cage or excited attempts to escape from the hand or the forceps, (3) squeaking, (4) urination and defecation. Although in some instances Coburn found almost complete dominance of wildness and savageness factors over factors for tameness, the results do not indicate any simple genetic principles of dominance and recessiveness. A certain amount of conditioning occurred during these experiments, since there is a decrease in the grades of wildness and savageness in successive tests. This decrease was undoubtedly brought about through handling and daily presence of the experimenter during routine care of the colony.

Savageness and wildness were observed by Yerkes (1913), Utsurikawa (1917), and Stone (1932) to occur coincidentally in rats. In each case the savageness and wildness were directed against the experimenter. Yerkes' results in the F_1 generation indicate that wildness and savageness are largely dominant over tameness. However, in the F_2 generation there was a decrease in savageness and wildness that cannot be explained on these grounds. Handling and unwitting selection of the F_1 generation for breeding may account for the results.

Inbred rats from the Wistar stock were found to differ from outbred stocks in exhibiting twice as much savageness as the outbred group. In this case, savageness was tested by recording the attempts to bite a copper wire thrust into the cage (Utsurikawa, 1917). Stone (1932), using an 11-point rating scale for wildness and savageness, found differences in wildness and savageness among wild rats, half-breeds, quarter-breeds, and tame rats, and he concluded that these differences are probably hereditary in nature. Information concerning other behavior studies may be found in a very extensive review of the literature on temperament in rats and mice which has been made by Hall (1941).

Androgens have been found to stimulate aggression in males and females of

many vertebrates. Since Collias (1944) has recently reviewed the literature in this field, only data pertaining to the effects of male hormone on aggressive behavior of males will be mentioned here.

Castrate male lizards, *Anolis carolinensis*, have been observed to fight more actively than controls after being injected with testosterone propionate (Noble and Greenberg, 1941). There is a large amount of evidence indicating that androgens increase aggressiveness in male birds. Testosterone propionate caused defense of territory in month-old male chicks of the black-crowned night heron (Noble and Wurm, 1940); and several investigators have been able to induce cocklike fighting in male chicks injected with androgens (see Noble and Zitrin, 1942). Three male valley quail, *Lophortyx californica vallicola*, which had been implanted with pellets of testosterone during the winter, became pugnacious toward other males and aggressive toward females within 45 hours after implantation. However, the position of these males in the social hierarchy did not change (Emlen and Lorenz, 1942). Male herring gulls which had been injected with testosterone propionate as embryos were, as juvenales the most aggressive birds in a colony maintained by Boss (1943) and dominated all the birds with which they came in contact.

Although Kochakian (1941) does not define aggressive behavior, he reports that castrate Rockland mice were aggressive after pellets of testosterone propionate had been implanted and that normal mice of this strain showed the same disposition. It is of interest to note that the Buffalo-Marsh mice used by Kochakian lacked these aggressive tendencies. After receiving methyl testosterone injections daily, the dominance status of a prepubertal castrate male chimpanzee was enhanced in a food-getting situation (Clark and Birch, 1945).

Unpublished data on a preliminary investigation by Allee and Warne indicate that when pellets of testosterone propionate were implanted in one Bagg albino and in four normal C57 black male mice, no unusual change in aggressive activity occurred. Two of these mice showed no aggressiveness, and the activity of the other three was within the normal range of variability of aggressiveness. These data are not out of line with those in the papers mentioned above, since in the other cases the androgen therapy was effective in inducing aggressiveness out-of-season, in castrates, or in immature animals.

In the present study one attack which was begun but not completed was the only sign of aggressive behavior which was noted on the first day after implantation of testosterone propionate. Two groups failed to exhibit aggressiveness on the second day following implantation. Aggression was exhibited within 4–6 days after implantation of testosterone propionate in seven previously unaggressive groups of C57 black and Bagg albino castrates. From the data of Kochakian (1941) it can be seen that 5 days after implantation of a 22-mg. pellet of testosterone propionate the combined weight of the seminal vesicles and prostate glands was less than one-third that of normal mice; 11 days after implantation of an 11-mg. pellet the weight was slightly less than two-thirds of the normal weight; and 13 days after the implantation of a 22-mg. pellet the glands had not yet attained full size. Since it has already been shown that the rate of absorption of testosterone propionate from pellets implanted in the present study is slightly greater than the rate of Kochakian's 11-mg. pellets and less than that of the 22-mg. pellets, it can be stated

that, in the present study, the mice began to display aggressive behavior at a time when the amount of hormone absorbed was not sufficient to stimulate the accessory glands to attain normal size.

Encounters occurred either 3 or 4 days after the removal of the androgen pellets. At this time and in the encounters which were staged during the next 3 weeks, the majority of the treated castrates did not display any aggression. Thus seven of the ten mice which experienced encounters after the removal of the androgen pellets failed to show aggressiveness; two displayed only 3 attacks, 4 tail rattles, and 1 mince in a total of twelve rounds; and the aggressiveness of the third mouse undoubtedly can be explained by incomplete removal of the androgen pellet.

Since the present experiments were not performed primarily to study the effects of castration and androgen therapy on the size of the accessory sex glands, data are not available on the weight of these glands in the days immediately following withdrawal of male hormone. However, other investigators have noted glandular changes as a result of the rapid disappearance of male hormone following castration. Deanesly and Parkes (1933) state that the seminal vesicles and the anterior prostatic lobe of mice lose about half their weight in 7 days; and Moore, Price, and Gallagher (1930) report changes in the middle and posterior lobes of the rat prostate, 4 days after castration: It is probable that the weights of accessory glands in the present experiment would approximate those of Deanesly and Parkes (1933). Moore, Price, and Gallagher (1930) also state that castration effects appear within the same time after cessation of injection of extracts of bull testes as after testes removal. In the present study the earliest encounters were staged 25 days after castration, and, at this time, the mice did not act aggressively. Experiments are now in progress to investigate the behavior of mice in encounters which occur immediately after castration.

There is evidence that aggressiveness by males may not be entirely dependent on androgens produced by the male gonad. Male swordtail fish will maintain position in their social orders from 1 to $6\frac{1}{2}$ months after castration (Noble and Borne, 1940). Male lizards which had been castrated for $1–4\frac{1}{2}$ months have been observed to display aggressive behavior, consisting of dewlap extension, flattening of sides, and fierce fighting with teeth—behavior precisely like that of normal males (Evans, 1936). Although gonadectomy in cocks results in a decrease in aggressiveness, fighting and aggressive behavior do occur in capons (Davis and Domm, 1943).

In some animals, mating behavior also persists for varying lengths of time after castration. Ability of male rats to copulate after castration at 90 days of age has been shown by Stone (1927) to disappear slowly. In his study, copulatory ability was retained for more than 7 months by two of forty-five gonadectomized males; by the end of the 8th month none of the rats displayed mating behavior. Moore (1939) concludes from previously unpublished data on rats and guinea pigs that copulatory ability may be expressed for long periods after castration, and it has been reported by Clark (1945) that the development of sexual behavior in the prepubertally castrated chimpanzee is similar to that in the normal animal.

The results of the present investigation on castration differ markedly from those of Uhrich (1938), who found that castration did not inhibit fighting in all mice. It is true that the experimental conditions of this study are not exactly

comparable with those used by Uhrich, since the mice in the present investigation were isolated except during encounters, which were staged on 2 days of each week, and thus could not form a true social organization such as Uhrich described for groups of caged mice. However, since Ginsburg and Allee (1942) indicate that mice kept in isolation are much more prone to exhibit aggressive activity when brought together for staged encounters than are mice which are constantly caged together, it might be expected that the mice of this study would exhibit more aggression than those used by Uhrich.

In attempting to account for the difference between the present results and those of Uhrich (1938), it has been necessary to analyze Uhrich's data in some detail. It is difficult to determine the exact experimental conditions experienced by Uhrich's mice, since the number of animals in each "group" is not given and the number of mice in each group that were castrates, unilateral castrates, and normals is not indicated.

Since Uhrich watched as many as thirty groups at once, it must have been impossible to record all the various aspects of aggressive behavior which appeared. It was not stated whether normal aspects of the aggressive behavior pattern other than fighting (e.g., tail-rattling, mincing, attacking, and parrying) were reduced in amount or were absent. In fact, fighting, identified as a mere snap or dart, on the one hand, or very severe biting, on the other, was the only type of aggression mentioned. "Attacking," as it is used in the present study, may possibly be included in his term "fighting."

Uhrich castrated some individuals in each of four groups before sexual maturity. Of these, two groups were composed solely of bilaterally castrated animals; two groups contained one or more males which retained at least one gonad. It is stated that fighting rarely occurred in three of these groups and that in one a unilateral castrate regularly routed another unilateral castrate. The unilateral castrates may be considered essentially normal, since Moore (1939) reports that, when one testis is removed, the remaining testis secretes a quantity of hormone normal for the former pair of testes. In two of the groups castrated before maturity, a mouse fought as late as 100 days after gonadectomy. From the data given it is impossible to determine, in the first case, which three of the four groups are meant and whether the two groups of animals in the latter case were composed completely of bilateral castrates.

In four additional groups which included some unilateral and some bilateral castrates, castration was performed after sexual maturity. In three of these, a bilateral castrate was exclusively dominant for some time. Castrates are stated to have fought as late as 156, 168, 206, 247, 255, and 292 days after castration. The accessory sexual organs of a male mouse which fought on the 144th day after castration were found to be in the typical castrate condition on that day, indicating that castration had been complete.

The difference between the results obtained by Uhrich and those presented here cannot be explained by assuming that, in the present study, a longer period of time had elapsed between castration and opportunity for aggression, since one set of mice was paired as early as 25 days after castration, the encounters of five sets were begun 40 days or less after gonadectomy, and all thirteen sets had begun encounters by 140 days after castration. Uhrich recorded fighting by six complete castrates at much later dates after castration.

The mice used in these tests cannot be considered to be too young or too old to

behave aggressively, since mice of comparable ages were actively aggressive. In Uhrich's colony, the minimum age at which the first fight was recorded in young mice was 30 days, and the average age of 40 groups of mice at the time of the first combat was 50 days. These figures may not be typical for the C57 black and Bagg albino males. However, it is of interest to note that, of the 13 sets of mice castrated, 4 sets were gonadectomized prior to 30 days, and 5 sets prior to 50 days of age. Thus, 30 of the 48 mice were not castrated until after 50 days of age, when aggressive behavior might normally have been expected to appear.

It is evident that there is a need for accurate definition of the stimuli which activate normal patterns of aggression in mice. Aggressive behavior has been analyzed for *Anolis carolinensis* in terms of a chain of reflexes (Evans, 1936); and overlapping stimulus-response components have been reported for the robin's territorial-defense reactions (Lack, 1939). In a study of the stimuli necessary for mating in rats, Beach (1942) reported that, for the majority of male rats lacking sexual experience, the receptive female is the only stimulus adequate to induce initial mating reactions. There are a few very excitable males, however, which will attempt to mate with animals other than the receptive female, and a small proportion of inexperienced males are not aroused to copulate even with a highly receptive female. In the same study Beach demonstrated that in sexually inexperienced copulators elimination of more than one of three sensory receptors (vision, olfaction, and cutaneous sensitivity in the snout and lips) was sufficient to prevent copulation in rats but that all sexually experienced males continued to copulate after any two of the three sensory receptors were destroyed. Tinbergen (1942) has reviewed additional studies concerned with releasive mechanisms in animals.

To the writer's knowledge, no comprehensive studies of this type have been made of the stimuli which are necessary to activate aggressive behavior in rodents. Since castrates did fight under the experimental conditions used by Uhrich (1938) and did not exhibit aggressiveness in the present study, it becomes of interest to attempt to determine the stimulus (or stimuli) adequate to elicit such behavior in mice.

This investigation has indicated that, without doubt, the castrate male is not an adequate stimulus to induce aggression among castrates. There is also evidence which suggests that a "normal-acting" male is not an adequate stimulus to induce aggression in gonadectomized males, since one castrate in C57 black Sets I and IX continued to act aggressively after removal of the androgen pellet and the five castrates which were members of these two groups did not exhibit aggressive behavior.

In making an analysis of Uhrich's data, we found that the fights by castrates which were specifically mentioned occurred when strange normal males were introduced or, in one instance, when a unilateral castrate was introduced with other members of his group. From the data given it cannot be ascertained whether these are the only conditions under which fighting by castrates occurred. It seems possible that castrates or normal animals which are members of a group continually caged together do not constitute adequate stimuli to elicit aggression in castrate males. At this time it is difficult to state what features of a newly introduced mouse are sufficient to identify a given individual as a stranger or what the sensory receptors are which enable identification to be made.

Personal observations support the

view that individual recognition among mice probably is very slight if not nonexistent. Uhrich (1938) believes that mice can clearly distinguish between an old member of a group and a newly introduced one but states: ". . . . it seems doubtful whether one mouse always distinguishes between other mice in the cage. Rather, it sometimes appears as though the subordinates do not recognize the dominant but merely flee from any mouse that happens to attack them or that assumes an aggressive attitude" (p. 402). Ginsburg and Allee (1942, p. 504) believe that it may be possible that "mice respond to deportment rather than to individuality." Evidence that vision is not essential in the reaction of albino mice to one another is given by Uhrich (1938). He found that mice maintained positions in their dominance hierarchy for some time after blinding and reacted to strange males in a manner similar to that of normal males.

Tinbergen (1942) states that internal factors which drive an animal to perform certain special actions may be of three different kinds: hormones, proprioceptive stimuli, and springs of impulses from within the central nervous system itself. Little is known about the effects of stimuli belonging to the last two categories on aggression in mice, but the possibility of their operation should not be overlooked.

SUMMARY

1. The aggressive behavior pattern of normal male C57 black mice is qualitatively similar to that of normal male Bagg albino mice.

2. Normal C57 black mice display more attacking and exhibit a general trend to fight more frequently than do the albino mice. The albino mice rattle their tails a greater number of times in each round than do the black mice.

3. When the ratio of

$$\frac{\text{Attacks}}{\text{Tail rattles} + \text{attacks}}$$

in both strains is compared, the difference between the two strains is highly significant; $P < 0.0001$ ($t = 11+$).

4. Fluctuations occur in the frequency with which the components of the aggressive behavior pattern are displayed by normal mice of both strains. Such changes may result from the initial aggressiveness of the group as a whole, conditioning the mice to show less aggression, and subsequent encounters with nonaggressive mice restoring the ability of mice to display aggressive behavior.

5. Similar variations in aggressiveness occur in androgen-implanted castrates.

6. As general aggressiveness increases, there is a decrease in grooming and vice versa.

7. Castration of C57 black and Bagg albino male mice as immature animals or as adults results in the failure of these mice to display aggressive behavior, regardless of age at isolation when a period of 25 or more days elapses between castration and initial encounters.

8. Testosterone propionate pellets subcutaneously implanted will induce castrate mice of both strains to display aggressive behavior characteristic in quality and quantity of normal male mice of comparable strain.

9. Removal of androgen pellets from castrates results in an almost immediate cessation of aggressiveness in the majority of mice.

10. After androgen therapy has begun, additional encounters may stimulate castrate mice to exhibit more aggressiveness within a single encounter, since any two mice which were members of sets of four mice displayed more tail-rattling, attack-

ing, and fighting in a given encounter than did mice which were members of sets of three.

11. The amount of aggressive behavior displayed is positively correlated with the size of the seminal vesicles and prostate glands, since both are affected by amount of androgen present.

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THE TRANSMISSION OF RADIO-STRONTIUM AND PLUTONIUM FROM MOTHER TO OFFSPRING IN LABORATORY ANIMALS

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THE program of the Health Division of the Manhattan Engineer District included investigations of the acute and of the late, or chronic, effects of exposure to the radioactive products of uranium fission and to plutonium (Pu^{239}), a transuranic, alpha-emitting element. Radio-strontium (Sr^{89}) was one of the widely used fission products. It emits a pure beta-ray with an energy of 1.5 mev, and it has a half-life of 55 days.

The following experiments were initially designed to determine (1) the effects of Sr^{89} and Pu on pregnancy and (2) the degree, if any, to which these materials are transferred to the offspring by way of the placenta and milk. The affirmative answers to the questions of placental and milk transmission led to further studies of the offspring with respect to the localization, retention, and possible harmful effects of Sr^{89} and Pu when administered to fetal and suckling animals. In two such experiments the young (at the time of writing) are now over 200 days old.

[1] This document is based on work performed under Contracts Nos. W-7401-eng-37 and W-31-109-eng-38 for the Manhattan Project at the Argonne National Laboratories.

MATERIALS AND METHODS

The animals employed were ABC mice, obtained from the Roscoe B. Jackson Memorial Laboratory, and Sprague-Dawley strain rats. Radio-strontium was given intraperitoneally to prospective rat and mouse mothers before or during pregnancy and to prepuberal mice by subcutaneous injection. Plutonium was administered intravenously preceding or during pregnancy. Supplementary data were obtained from a pregnant dog that received Sr^{89} intramuscularly and from a lactating cat that received Pu intraperitoneally.

Placental transmission was measured by killing one member of each litter within 24 hours of birth and determining the microcuries (μc) of Sr^{89} or of Pu which it

contained.[2] The amount of a radioactive element which is present in any animal, in any part of an animal, or in a solution is referred to as its "activity." The "specific activity" (activity per gram of body weight) was used, since it was found to give a more consistent estimate of the activity of littermates (coefficient of variation = 7.6 per cent) than did the total activity (coefficient of variation = 12.6 per cent). Milk transmission and the retention of placentally administered materials were measured by exchanging offspring of control mothers with offspring of treated mothers.

Littermates were weighed together every 5 days until they were 30 days old; individual weights were taken thereafter every 10 days until the animals were 60 days of age and at monthly intervals subsequently.

Mothers that were treated with Sr^{89} were measured for their radioactivity within 24 hours (and usually within 12 hours) of parturition by means of the "external-count" method. This method involves caging the living animal in a cellulose acetate container (thickness, 0.18 mm.) that prohibits movement and placing it under a Geiger-Mueller tube. The discharges per second are converted to microcuries by means of factors that have been determined by correlating the external counts and ash counts of a large series of animals. Some of the factors that affect this determination are the thickness of the cellulose acetate cage, the distance between the animal and the tube, and the weight of the animal. Although several errors are involved in the method, it gives a reasonably reliable estimate of the Sr^{89} content of living animals.

[2] The author is indebted to Mr. Walter Kisieleski and to Miss Lois Woodruff for the analysis of those animals that contained Pu.

EFFECT OF SR^{89} AND PU ON MOTHERS

PREGNANCY AND PARTURITION

Ninety-eight young adult female mice were permitted to mate after having received intraperitoneal injections of from 0.1 to 5.0 μc/gm of Sr^{89}. Their litters were born 20–97 days after treatment. A decrease in the number of litters produced at the 5.0- and 2.5-μc/gm levels was observed; litter size was unaffected in those that delivered (Table 1). The high incidence of stillborn deliveries at 0.01 μc/gm and of stillborn young within viable litters at 0.5 μc/gm was probably accidental.

Treatment with Sr^{89} during pregnancy with doses acutely lethal to nonpregnant animals (10 and 5 μc/gm) greatly increased the percentage of totally stillborn deliveries, as well as the number of stillborn young among viable litters. A high incidence of totally stillborn deliveries likewise occurred after the injection of 0.06 μc/gm of Pu, although there were no stillborn young among any of the viable litters at this level. However, in the two lower levels (0.03 and 0.016 μc/gm) the percentages of stillbirths among viable litters were greater than in the controls.

SURVIVAL

Since it is conceivable that radiation damage might be less severe in mothers than in virgin animals because of the possible removal of Sr^{89} and Pu by pregnancy and lactation, comparisons were made between the percentages of mothers, nonmothers of the present experiments, and nonmothers of other experiments (Brues *et al.*) that survived 200 days after the administration of Pu and 220 days after the administration of Sr^{89} (Table 2). The only apparent beneficial

effect of motherhood among the Sr-treated animals was longevity—those that received a highly lethal dose (10 μc/gm) lived an average of 60 days, whereas nonpregnant animals similarly treated lived from 10 to 12 days. One mother died 214 days after treatment, and another was still alive at 220 days. In the Pu series it appeared that motherhood not only increased life-expectancy but also decreased radiation damage. In other experiments on nonpregnant mice, with the same dose levels and mode of administration, one osteogenic sarcoma and many instances of liver damage were noted by 200 days. No similar pathological conditions have been demonstrated to date in any of the mothers.

TABLE 1

THE EFFECT OF Sr^{89} AND Pu ON PREGNANCY AND PARTURITION

Dose Level (μc/Gm)	Species	Number of Animals	Animals Which Delivered (%)	Deliveries Which Were Totally Stillborn (% of Total Deliveries)	Viable Litters with One or More Stillborn Individuals (% of Viable Litters)	Average Litter Size (No. of Animals)
	Controls Injected before or during Pregnancy					
0 (Sr)	Mice	102	82	7	4.6	7.4
0 (Pu)	Mice	40	85	0.7	5.8	8.1
0 (Pu)	Rats	18	86.7	.....	.....	8.4
	Sr^{89} Injected before Conception					
5.0	Mice	8	2.5	0	0	7.0
2.5	Mice	10	50	0	0	7.2
1.0	Mice	10	90	0	0	6.0
0.5	Mice	10	100	0	40	6.6
0.25	Mice	10	90	0	11	6.4
0.2	Mice	10	90	0	0	6.8
0.1	Mice	10	100	10	0	6.6
0.05	Mice	10	100	10	0	5.5
0.02	Mice	10	70	14	0	6.7
0.01	Mice	10	100	30	0	7.0
	Sr^{89} Injected during Pregnancy					
10.0	Mice	20	85	23.5	23	6.8
5.0	Mice	20	85	11.8	20	7.6
2.5	Mice	20	100	5.0	5	7.9
	Pu Injected Shortly before or during Pregnancy					
0.06	Mice	20	85	23.5	0	7.1
0.03	Mice	20	85	0	17.6	6.5
0.016	Mice	20	100	0	25	7.7
0.016–0.03	Rats	15	86.7	.....	.....	7.8

STUDIES OF OFFSPRING TREATED WITH Sr^{89}

The animals used in this series consist of the litters of (*a*) 9 rats which received 0.7–2.0 μc/gm of Sr^{89} during late pregnancy; (*b*) 105 mice which received 0.01–5.0 μc/gm before conception; (*c*) 75 mice which received 2.5–10.0 μc/gm during pregnancy; and (*d*) 110 control mice which received injections of 0.2 ml. Ringer's solution before or during pregnancy. In addition, 117 young mice were given subcutaneous injections of 1.0–10.0 μc per animal at 5, 15, or 25 days of age.

DOSE ADMINISTERED TO YOUNG

Placental transmission.—Radio-strontium readily passes from mother to fetus. The total amount of Sr^{89} found in the young at birth varied directly with their weights[3] (Table 3), directly with the

[3] The average birth weights were: mice, 1.3 gm.; rats, 5.7 gm.

TABLE 2

SURVIVAL OF FEMALE ABC MICE TREATED WITH Sr^{89} AND Pu

Dose Level (μc/Gm)	Numbers Alive 200 Days (Pu) and 220 Days (Sr) after Injection					
	Other Experiments*		Nonmothers		Mothers	
	Total Number of Animals	Percentage Survival	Total Number of Animals	Percentage Survival	Total Number of Animals	Percentage Survival
	Sr^{89}					
10.0	>10	0	3	0	17	6
5.0	15	45	3	0	17	29
2.5	19	85	0	.....	20	95
	Pu					
0.06	40	0	3	0	17	18
0.03	32	31	3	0	17	59
0.016	40	70	0	.....	20	100

* Data from experiments in progress.

TABLE 3

THE RELATION BETWEEN WEIGHT AT BIRTH AND TOTAL ACTIVITY AMONG LITTERMATES

Litter	Number of Animals	Mean Sr^{89} Content (μc/Mouse)	Coefficient of Variation	Mean Sr^{89} Concentration (μc/Gm)	Coefficient of Variation
1	8	0.059	10.2	0.047	10.6
2	6	0.098	19.4	0.079	7.6
3	8	0.058	8.6	0.046	6.5
4	10	0.037	8.1	0.036	5.6
Mean	.....	.....	12.6	.....	7.6

amount administered to the mother (Fig. 1), and inversely with the length of time from treatment of the mother to delivery, when that time exceeded 3 days (Fig. 1). The ratio of the specific activity of the young at birth to the specific activity of the mother (either at injection or at delivery) was independent of the dose level (Table 4) but was dependent upon the number of days between injection and delivery (Fig. 2). The specific activity of the young, born 1–4 days after treatment of the mother, exceeded that of the mother at the time of parturition. The ratio decreased rapidly during the period of from 5 to 20 days and then leveled off at about 7 per cent. The difference between this ratio and the ratio

$$\frac{\mu c/gm \text{ young at birth}}{\mu c/gm \text{ injected into mother}}$$

is primarily due to the loss of Sr^{89} from the mother. Although the latter ratio is useful in predicting the amount of activity that will be found in any litter, the former, by expressing the relation between the concentration of Sr^{89} in the young and that in the mother at the time of delivery, gives a more illuminating picture of placental transfer.

TABLE 4

A REPRESENTATIVE SAMPLE OF THE RATIO

$$\frac{\mu c/gm \text{ young at birth}}{\mu c/gm \text{ mother at delivery}}$$

Injection-Delivery Interval (Days)	Original Dose to Mother (μc)	Ratio
1	200	2.39
	100	2.39
	50	2.24
	50	1.85
2	200	2.14
	100	2.18
	100	1.81
	50	1.74
3	200	1.70
	200	1.80
	100	2.78
	100	1.94
	50	1.81
	50	2.18
4	200	1.30
	100	1.29
	50	1.56
	50	1.35

The underlying factors responsible for the curve in Figure 2 are difficult to ascertain. It appears that the uptake of Sr^{89} by the fetus is greatest during the last days of gestation (19th–20th days)

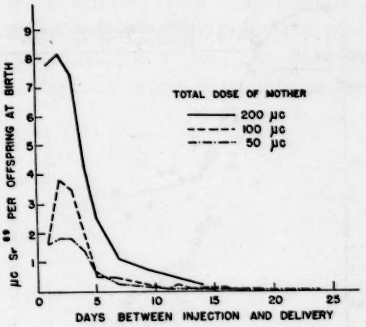

FIG. 1.—The total Sr^{89} content per offspring at birth varies with the original dose of the mother and with the injection-delivery interval.

and probably is negligible before the 15th day. Pecher and Pecher (1941) did not find Sr^{89} in mouse fetuses before the third quarter of gestation. The leveling of the curve at about 7 per cent occurred when treatment preceded conception, and this value remained relatively constant, even though the Sr^{89} content of the maternal blood continued to fall (Anthony *et al.*). The data at hand suggest that the amount of Sr^{89} appearing in the offspring that are born 20 or more days after treatment of the mother is dependent upon the amount of radioactive material present in the mother's bones. This relationship is not unlikely, since it is known that calcium is removed from trabecular bone during pregnancy.

Contrary to expectation, it was found

that, when the time from injection to delivery exceeded 1 day, the amount of Sr^{89} retained by the mothers immediately after parturition closely approximated the retention by nonpregnant animals (Anthony *et al.*) (Table 5). It is interesting to note that in the nonpregnant animals Sr^{89} is lost via the urine and feces, while in the pregnant animals as much as 75–80 per cent of the total amount lost

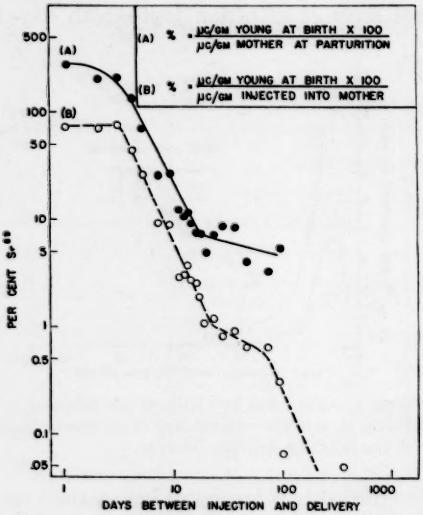

FIG. 2.—Placental transmission of Sr^{89}. The relationship between the concentration of Sr^{89} in mother and offspring is dependent upon the injection-delivery interval. It is independent of the dose of the mother.

was found in the litters (Fig. 3). The values declined sharply with increase in the injection-delivery interval; and, when this period exceeded 5 days, they fell slowly from the 10 per cent level. These values do not take into account the decay of Sr^{89} or the amounts of the isotope lost in the amniotic fluid and in the placental tissues, which in the dog were found to comprise 1 per cent of the total present in the intact fetus.

Milk transmission.—Ten normal litters of mice (a total of 83 animals) were nursed from birth to weaning by foster-mothers, each of which had received a total of 100 μc of Sr^{89} during pregnancy. The total activity of each foster-mother at the time that she delivered and received the normal litter was estimated by the external-count method. The foster-mothers fell into three groups, with values clustering about estimated total microcurie averages of 65, 41, and 17.

TABLE 5

COMPARISON OF THE RETENTION OF Sr^{89} BY PREGNANT AND NONPREGNANT ADULT MICE

Days after Injection	Percentage Retention by Pregnant Mice	Percentage Retention by Nonpregnant Mice*
1	33	55
2	48	48
3	51	45
4	44	43
5	40	41
7	41	38
9	29	37
11	37	35
12	38	34
13	36	33
14	32	32
16	36	31
17	34	30
19	29	29

* Data from Anthony *et al.*

One to three animals from each litter were analyzed at 15, 25, 35, and 45 days of age. Figure 4 shows the average total Sr^{89} content of the young mice. It was expected that the young which were nursed by the 41- and 65-μc mothers would contain about two and four times as much activity, respectively, as those nursed by the 17-μc mothers. Instead, only 1.15 and 1.35 times as much Sr^{89} was recovered in the young at 15 days. There was less Sr^{89} in the young at 25 days

than there was at 15 days. Cow's milk and bread were constantly available in the cages, and at about 15 days of age the young mice started weaning. Few animals were found to be breast-feeding after 25 days; hence, the curves beyond the 15th day are primarily retention curves.

Double-log representation of the percentage of the foster-mother's total activity at parturition which was found in the young at various ages, plotted against the total activity of the foster-mother at parturition, produces approximately straight lines for the several ages studied (Fig. 5). The striking inverse relationship between the percentage of Sr^{89} that was recovered from the young and the total activity of the foster-mother is again demonstrated.

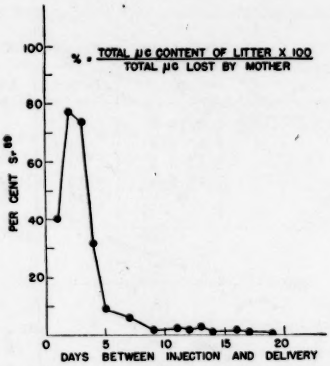

FIG. 3.—The relationship between the Sr^{89} that was lost by the mother between treatment and parturition and the Sr^{89} that was present in her litter at birth varies with the injection-delivery interval.

The total 15-day dose.—The great variation in the amount of placentally administered Sr^{89}, which was dependent upon the length of time between treatment of the mother and parturition, and the additional variation in the dose derived from the milk made it necessary to regroup the litters of the long-range mouse experiment on the basis of their estimated dose at a given age rather than upon the original dose delivered to the mothers. The total dose at 15 days of age was selected as a standard, since at that time the milk-administered Sr^{89} is maximal. The 15-day dose is readily calculated by correcting the Sr^{89} content at birth for retention (see below, p. 413) and adding the activity derived from the milk, which amount can be determined from Figure 5.

The total Sr^{89} content of the 15-day-old mice ranged from 0.2 to 10.6 μc. Since the weight of a 15-day-old mouse is approximately 7 gm., the dose level at that age, in terms of specific activity

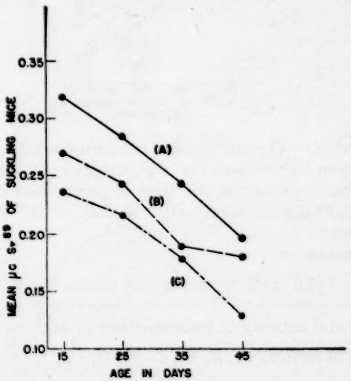

FIG. 4.—Milk transmission of Sr^{89}. The mean Sr^{89} content (in μc) of suckling mice varies with age and with the dose level of the foster-mother. Average μc content of foster-mother at delivery: *A*, 65; *B*, 41; *C*, 17.

varied from 0.03 to 1.5 μc/gm. The litters were arranged in arbitrary groups (Table 6) that form the basis of classification of the long-range effects as they are being followed throughout the lives of the mice. Although there was a correlation between the dose originally given to the mother and the group to which her litter was assigned, the length of time between injection and delivery proved to be equally important. All the litters of

mothers that received a total of 200 μc fell into Group I, with the exception of one that was assigned to Group III. Mothers treated with 100 μc contributed four litters to Group II, two to Group III, and six to Group IV. The 50-μc mothers contributed ten litters to Group III and seven to Group IV. The greater accuracy obtained by this regrouping was demonstrated by the fact that the standard deviations, and hence the coefficients of variation, of the weights after the 10th day were considerably smaller than they had been when the original dose level of the mother determined the group.

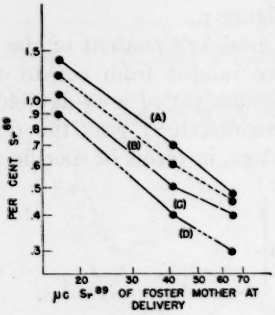

FIG. 5.—The relationship between the mammary secretion of Sr^{89} and the microcuries contained by the foster-mother at the time of parturition shows a negative correlation with the dose of the foster-mother.

Percentage =

$$\frac{\text{Total activity of suckling mouse} \times 100}{\text{Total activity of foster-mother at delivery}}$$

Days of age: *A*, 15; *B*, 25; *C*, 35; *D*, 45.

The relative importance of the Sr^{89} transmitted through the placenta and that transmitted through the milk is illustrated in Figure 6. When injection preceded delivery by less than $7\frac{1}{2}$ days, more than 50 per cent of the 15-day dose came through the placenta; when the interval exceeded $7\frac{1}{2}$ days, more than 50 per cent was derived from the milk.

RETENTION

The metabolic similarities of Ca and Sr^{89} suggested that the young growing animal might show a higher retention of Sr^{89} than would the adult animal. This was verified by a study of thirty-five litters of mice. Ten litters received the active material through the placenta alone, and ten through nursing alone. Five litters were given subcutaneous injections at 5 days of age, five at 15 days of age, and five at 25 days of age. The total Sr^{89} content of one to three animals of each litter was determined at regular intervals; decay corrections were made for the time from administration (or birth, in the case of those animals which received the Sr^{89} *in utero*) to analysis. A comparison of the retention curves in Figure 7 shows that the younger the animal is at the time of treatment, the

TABLE 6

Sr^{89} CONTENT AT 15 DAYS OF AGE

Group	Sr^{89} Content at 15 Days (μc)	Number of Litters	Number of Animals
I	7.0–10.6	5	36
II	4.4– 5.2	4	32
III	1.1– 2.7	13	100
IV	0.2– 0.9	13	110
Controls	0	26	192

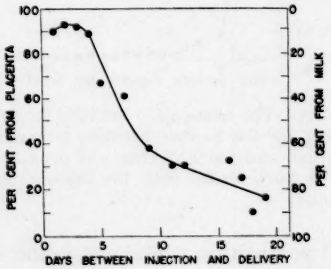

FIG. 6.—The relative contributions of placentally and milk-administered Sr^{89} varies with the injection-delivery interval.

greater is its retention of Sr^{89}. The level of the curve is largely determined by the loss through excretion that occurs during the first 5 days after administration; the slope of the curve after this initial excretion is relatively constant, regardless of the age of the animal.

Two mice from the previously described Groups II and III were analyzed at about 200 days of age and were found to contain 18.3 and 18.8 per cent, respectively, of their 15-day doses. In contrast, animals that were adults when treated were found to contain only 2 per cent of their original doses 200 days later. These are absolute recoveries not corrected for decay.

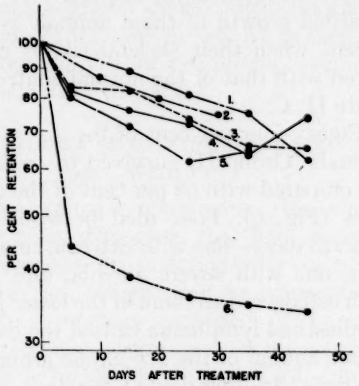

FIG. 7.—The retention of Sr^{89} by young and adult mice.

No.	Age at Injection	Mode of Administration
1	Fetus	Placenta
2	1–15 days	Milk
3	5 days	Subcutaneous
4	15 days	Subcutaneous
5	25 days	Subcutaneous
6	Adult	Intraperitoneal

EFFECTS ON YOUNG

Placentally administered Sr^{89} is heavily concentrated in the ossifying bones of newborn mice. Radio-autographs clearly outline the skull, jaws, vertebral column, and sternum (Pl. I, *A*, *B*). At 16 days of age the center of activity in the long bones is located in the shaft (Pl. I, *C*). These facts suggest that the greatest evidences of radiation damage might appear in bone formation, skeletal growth, and hemopoiesis.

Body weight was used as an indicator of growth rate. In the mice, weight differences between Group I (7.0–10.6 μc) and the other levels were detectable as early as 5 days of age and were very pronounced by 10 days (Fig. 8). Group II animals (4.4–5.2 μc) fell below the lower doses and controls by the 10th day, and at 210 days their average weight was about half that of the controls. Weights of Groups III (1.1–2.7 μc) and IV (0.2–0.9 μc) were about 90 per cent and 95 per cent, respectively, of control weights after 50 days of age. In the rats, which were weighed every 2 weeks from 40 to

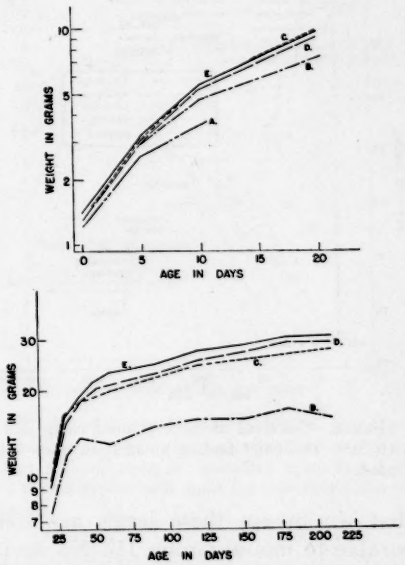

FIG. 8.—The effect of Sr^{89} on the growth rate of mice as indicated by increase in weight. Radiostrontium content of mice at 15 days of age: *A*, 7.0–10.6 μc; *B*, 4.4–5.2 μc; *C*, 1.1–2.7 μc; *D*, 0.2–0.9 μc; *E*, controls.

175 days of age, no difference occurred between those that contained 2.5 μc at birth and the controls. However, those that contained an average of 6.5 μc consistently weighed 20 per cent less than the controls.

Periodic blood studies have been made only on the rats, which had a total activity at birth of from 2 to 8 μc. Since the weight of an adult rat is about ten times that of a mouse, these levels are comparable to mouse Group IV. No deviations from normal blood counts were noted during the observation period (40–180 days of age). Blood samples are being taken on all moribund mice; the results of this study will be reported at a later date.

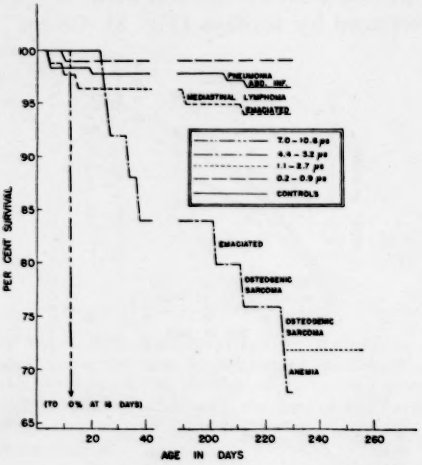

FIG. 9.—Survival of mice treated early in life with Sr^{89}. The chief finding at each autopsy is included.

Radiation damage was apparent in Group I mice as early as the 10th day. The animals were underweight, inactive, and pale, and hair failed to appear in a line from eye to ear. (Generalized sparseness of hair on the head and face was observed in one litter belonging to Group III; however, by 50 days these animals were normal in appearance.) All the animals of Group I were dead by the 16th day. Group II mice were noticeably stunted by the 15th day; only their feet seemed to have grown at the normal rate. By 30 days their long bones were fragile, bent, and noticeably shortened. Plate II, *A*, *B*, illustrates this type of bone malformation in animals that died at 325 and 309 days, respectively. The retarded growth of these animals is apparent when their skeletal size is compared with that of the normal control in Plate II, *C*.

Eighty-four per cent of the 4.4–5.4-μc animals (Group II) survived to 40 days, as compared with 98 per cent of the controls (Fig. 9). Four died between 200 and 228 days—one with extreme emaciation, one with severe anemia, and two with osteogenic sarcoma of the lower jaw. Mediastinal lymphoma caused the death of one animal of the 1.1–2.8-μc group at 190 days. Another died at 212 days, with no gross lesions; however, it was extremely emaciated. Two control animals died between 204 and 212 days—one had pneumonia, and the other suffered from an unidentified abdominal infection. A fibrosarcoma in the region of the right tibia appeared at 240 days in a rat that had a total of 5.8 μc at birth.

The study of a litter of puppies, which were born 6 days after the mother had received 1μc/gm of Sr^{89}, has added some interesting information with respect to the effect of the isotope upon very young animals. Close to 300 μc were found in one puppy that was analyzed at birth. This gives a specific activity of 1.5 μc/gm, which value is above the median lethal dose for adult dogs (Prosser and Swift). The four remaining puppies were placed on formula feeding at 4 days of age because of the poor condition of the mother, and they were apparently doing well until the death of one at 18 days. No

gross lesions were found in any of the tissues, and the cause of death remains unknown. The puppies were able to stand and take a few steps on the 20th day, and by the 26th day one walked with ease and romped about the cage. However, by the 36th day none were able to get up on all four feet, although they appeared normal in other respects. Roentgenograms showed deficient calcification of the long bones. At 40 days of age the puppies showed no improvement in the ability to walk.

STUDIES OF OFFSPRING TREATED WITH PU

The animals comprising this study were born of mothers that had received intravenous Pu injections during pregnancy or shortly before conception. Treated mothers consisted of 15 rats (0.03–0.0016 μc/gm) and 60 mice (0.06–0.016 μc/gm). The litters of 18 control rats and 40 control mice completed the series.

DOSE ADMINISTERED TO YOUNG

Placental transmission.—A very small percentage of the Pu injected into pregnant animals was found in the offspring at birth. Whether this was the result of low retention by the fetus or to low transmission by the placenta is not known, but the latter alternative appears more probable. The Pu content per gram of body weight of the young at birth expressed as the percentage of that injected into the mother per gram of body weight was dependent upon the number of days between injection and delivery (Fig. 10), in which respect it is similar to the situation found for placental transmission of Sr^{89}. The Pu data differ from the Sr^{89} data in one important respect. It has been seen that the Sr^{89} ratio is independent of the original dose given to the mother; however, the Pu ratio varies inversely with the original dose. The specific concentration in the young at birth is about 1.5 per cent of that injected into the mother when the mother received 0.06 μc/gm; 3 per cent when the mother received 0.03 μc/gm; and 6 per cent when the mother received 0.016 μc/gm.

Milk transmission.—An experiment primarily designed to discover the amount of Pu that is transferred to nursing young by treated mothers is in only preliminary stages. However, the scattered data that are now available demonstrate that Pu is secreted by the mammary glands and that a portion of that ingested by the young animal is absorbed. Nine suckling mice were analyzed between 8 and 20 days of age, and in each instance their Pu content exceeded that of littermates at birth. Two normal rats were nursed from birth by a foster-mother that had previously received 6.25 μc. The Pu content of each at the time of sacrifice was as follows: 17 days—

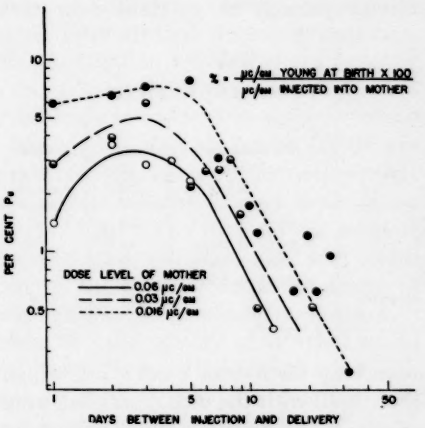

FIG. 10.—Placental transmission of Pu. The relationship between the concentration of Pu in mother and offspring is dependent upon the dose level of the mother and upon the injection-delivery interval.

0.004 μc; 33 days—0.0013 μc. Unfortunately, the milk-filled stomach of the 17-day old animal was not analyzed separately; at 33 days this difficulty did not arise, since breast-feeding had ceased. Additional information was supplied by three rats which were delivered and nursed by the foster-mother used above. Their Pu content was as follows: 0 days —0.0008 μc; 17 days—0.003 μc; 33 days—0.0019 μc. The increase in the activity present at 33 days over that present at birth was 0.0011 μc, which agreed well with the 0.0013 μc that were present in the control animal nursed by the same treated mother and sacrificed at the same age.

TABLE 7

PU CONTENT AT BIRTH

Group	Pu Content at Birth (μc)	Number of Litters	Number of Animals
I.....	0.0019–0.0028	8	58
II.....	0.0013–0.0018	15	118
III.....	0.0007–0.0011	6	40
IV.....	0.0001–0.0005	15	102
Controls..	0	34	276

Plutonium has been directly demonstrated in the milk of a lactating cat that had received an intraperitoneal injection of 0.097 mc (0.03 μc/gm) 8 days after parturition. Nine days later the breast milk contained 0.0035 μc/ml. One kitten was sacrificed at 24 days of age, or after it had received Pu-containing milk for 16 days. Approximately 0.9 μc were present in the alimentary canal and 0.19 μc in all other tissues.

The "total" dose.—Until adequate data on the milk transmission of Pu are obtained, the total dose to which the mice of the long-term experiment have been exposed cannot be determined. The litters have been arranged tentatively on the basis of their zero-day dose alone, as determined by the analyses of littermates sacrificed at birth (Table 7). The dose levels are extremely low, but they are not negligible, in view of the fact that the milk-administered dose may equal or even exceed the placentally administered dose and that retention appears to be close to 100 per cent, in contrast to the 50 per cent retention determined for adult animals (Snyder *et al.*). The data supporting this view are meager but nonetheless indicative. For example, two rats born of a treated mother and nursed by a normal foster-mother contained the same amounts of Pu at 17 days (0.0009 μc) and at 33 days (0.0008 μc) as did a littermate at birth (0.0008 μc).

EFFECTS ON YOUNG

The body weights of the Pu-treated mice did not differ significantly from those of controls (Fig. 11). The average weight of Group I mice consistently fell slightly below controls; Group II mice weighed less than the controls up to 60 days and equaled the controls thereafter; the weights of Groups III and IV exceeded those of the controls after 60 days. Whether these differences are sig-

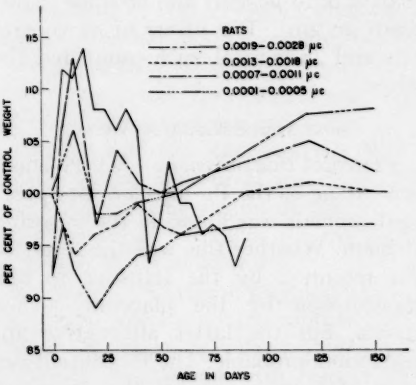

FIG. 11.—The effect of Pu on the growth rate of rats and mice as indicated by the comparison of the weights of treated and control animals.

nificant will depend upon the analyses of the weights beyond 150 days. The treated rats, on the other hand, showed a possibly increased growth rate over the controls during the first 40 days. From 0 to 4 days their weights did not differ from control weights, but during the period from 5 to 40 days the treated animals were 10 per cent heavier than the control animals. The *t*-test applied to this portion of the curve gives a probability value of less than 1 per cent that this difference is due to chance. The Pu content of the rats at birth was 0.00045 μc; and, in view of the fact that the newborn rats weighed four to five times as much as the newborn mice, the dose level of the rats (in μc/gm) was below that of the Group IV mice. Further work is necessary before it can be said that very low doses of Pu accelerate early growth.

No radiation damage has been detected in any of the Pu-treated animals. However, the animals are only 200 days old at this writing, and latent effects at low levels in other experiments dealing with adult mice did not appear until 350 days after treatment (Brues *et al.*). The few deaths that have occurred cannot be attributed to Pu poisoning. The percentages of the animals alive at 1 day that are still living at 200 days are as follows: Group I, 100 per cent; Group II, 99 per cent; Group III, 97 per cent; Group IV, 94 per cent; and controls, 96 per cent.

DISCUSSION

The deleterious effects of Sr^{89} and Pu on pregnancy were shown by (1) the low incidence of conception when the administration of 2.5 or 5 μc of Sr^{89} per gram of body weight preceded mating and (2) the high percentage of totally stillborn litters and litters with one or more stillborn individuals when 5–10 μc/gm of Sr^{89} or 0.016–0.06 μc/gm of Pu was administered during gestation. There was no evidence of total or partial litter resorption, since every diagnosed pregnancy was followed by parturition, and the average litter size of the treated animals did not differ significantly from that of the controls. It is possible that the failure to become pregnant when treatment preceded the opportunity to mate was the result of a cessation of the oestrous cycle. Whether the high incidence of stillbirths among the animals which were treated during gestation is due to radiation damage of the fetus or is the indirect result of maternal damage is not known.

There is no obvious reason for the remarkable survival of those mothers that received the highly lethal dose of 10 μc/gm of Sr^{89}. The data do not support the original expectation that the retention of the isotope would be lower in mothers than in nonmothers. Instead, it seems that the amount of Sr^{89} that would ordinarily be excreted by an adult animal is divided into one fraction that passes through the placenta and is retained by the fetus and another fraction that is excreted. The proportion of these two varies with the period of gestation at the time of treatment; i.e., about 80 per cent of the amount lost by the mother is found in her litter if injection precedes delivery by 2 days, while less than 10 per cent is detected in the litter if injection precedes delivery by more than 6 days.

It is likewise difficult to explain the fact that among the Pu-treated mothers there was a larger percentage alive at 200 days than would be expected on the basis of other experiments. Although very little of the material is transferred to the fetus by way of the placenta, it is possible that a relatively larger amount is secreted by the mammary glands. Further investigation along this line is

necessary before the prolonged life-span of these mothers can be attributed to Pu removal.

The Sr^{89} and Pu mothers that lived to 220 and 200 days, respectively, showed typical late-radiation symptoms, with the exception that bone tumors and Pu-induced liver damage, which occurred at about this time in other experiments, have not yet appeared in these. Many of the living animals have grayed coats, and they are anemic and emaciated.

The percentage of the maternal radioactive material that passes through the placenta and is retained by the fetus is independent of the dose level when Sr^{89} is used and is inversely proportional to the dose level when Pu is used. Why the latter situation should obtain is not clear. One possible explanation is that placental functions are damaged by Pu, and the greater the amount to which the placenta is exposed, the smaller the relative amount it passes on to the fetus. However, since the birth weights of the Pu-treated young do not differ from those of the controls, placental dysfunction is not evident. Perhaps the answer lies in the immediate distribution of the material within the mothers at the various dose levels; e.g., at the 0.06-μc/gm level there may be a smaller relative amount of Pu in the circulating blood than at the 0.03-μc/gm level.

The slopes of the Pu and Sr^{89} curves are fundamentally the same when the ratio, "specific activity of young to specific activity of mother," is plotted against the number of days between injection and delivery. These two curves, as well as the curve representing the percentage of the total Sr^{89} lost by the mother that was recovered in her litter, indicate that fetal assimilation of these materials is greatest during the last 4 or 5 days of gestation and is probably negligible before that time.

There are two apparent explanations for the fact that the higher the total activity of the lactating animals, the lower the relative amount of Sr^{89} recovered from the young which they have nursed: (1) The absorption and assimilation of Sr^{89} may have been proportionately lower in those young that had a larger total amount available to them. This, however, does not seem likely. (2) Generalized radiation injury of the mother or damage of the mammary glands in particular may have led to decreased milk production. By 15 days of age (before weaning) the foster-litters of the mothers that contained 17 μc at parturition weighed 7.4 gm.; the foster-litters of those that contained 41 μc weighed 6.7 gm.; and the foster-litters of those that contained 61 μc weighed only 5.3 gm. These data indicate a progressively smaller milk supply as the dose level of the mother increased, since the actual Sr^{89} content of the young was too low to account for any weight differences. The radiation to which the mammary tissue itself had been exposed is difficult to ascertain. If we assume (1) that Sr^{89} is utilized in the same fashion as is Ca; (2) that infant mice absorb about 50 per cent of the Ca content of breast milk, as do human infants; and (3) that an average-sized litter is nursed (seven to eight young), the observed recovery of 0.3 μc of Sr^{89} in a 15-day-old animal would indicate that, during the first 15 days of lactation, the mammary tissue of the foster-mother had been exposed to 4–5 μc, or had secreted about 10 per cent of her parturition-day dose. Analysis of the breast milk and the blood of a dog that had received 1 mc/kg, 6 days before parturition, showed that the

specific activity of the milk was ten times as great as that of the plasma.

It has been found that only a very small fraction of the Pu introduced into the stomachs of adult rats and mice is absorbed (Snyder *et al.*). However, there is unquestionable evidence that young animals obtain a considerable quantity of Pu from the milk of their treated mothers; for instance, 0.19 μc was recovered from the extra-alimentary tissues of a kitten that had ingested breast milk containing 0.0035 μc/ml for 16 days. The kitten gained 120 gm. during the experimental period, or 0.0016 μc with each gram of body weight gained. There may be a real difference in intestinal permeability to Pu in young and adult animals, such as has been postulated for the absorption of some viruses and for the cancer-producing substance transmitted by lactating mice. An alternative hypothesis is that the Pu is transformed into an absorbable complex as it becomes a constituent of the milk.

The deleterious effects of the early treatment with Sr^{89} are manifested by (1) death by the 16th day of age at high-dose levels, (2) retardation of growth at all levels studied, and (3) the appearance of late radiation symptoms, such as anemia and bone tumors, at intermediate levels. It may be argued that the acute deaths resulted from the failure of the mothers to produce milk and/or care for their young. However, there is a much stronger correlation between the presence of 7–10 μc at birth and death by the 16th day than there is between the parturition-day radioactivity of the mother and the early death of her litter. The retardation of growth likewise might be attributed to malnutrition. Although this explanation may partially account for the differences among the early weights, it must be borne in mind that cow's milk and bread, which were constantly present in the cages, were readily eaten by the young mice after their 15th day. Here again the correlation between rate of growth and dose level at birth is much greater than that between rate of growth and parturition-day dose of the mother. Two likely explanations for the growth effects—one or both of which may prove to be correct after further experimentation—are: (1) damage to the young bones by their heavy concentration of Sr^{89} and (2) damage to the pituitary gland, with subsequent metabolic effects, since the situation of the gland within the sella turcica subjects it to considerable irradiation.

The first bone tumor among the mice treated before birth with Sr^{89} occurred when the animal was 210 days old. The earliest bone tumor recorded in other experiments employing adult mice appeared 182 days after treatment, at which time the animal was about 240 days old. Statistically valid numbers of animals will have to be available before any definite statements can be made; but, at the present stage of our information from these and other experiments, it appears that the important time factor in tumor formation is not the absolute age of the animal but rather the length of time after treatment. These data also suggest that embryonic and young tissues are neither more nor less sensitive to Sr^{89} than are adult tissues.

No Pu damage has been noted to date among the animals that received the material from their mothers. Whether they will develop abnormalities similar to those observed in the animals treated when they were adults (e.g., tumors and liver damage) remains to be seen. There was a latent period of about 350 days in the low-dose adult experiments; only 200

days have elapsed since the birth of the animals whose treatment began *in utero*.

SUMMARY

1. Mice which were injected with 5.0 or 2.5 μc/gm of Sr^{89} before conception produced a smaller number of litters than did the controls. Treatment during pregnancy with 10 or 5 μc/gm of Sr^{89} or 0.06, 0.03, or 0.016 μc/gm of plutonium increased the percentage of totally stillborn deliveries, as well as the number of stillborn young in viable litters.

2. The mice which were treated during pregnancy with either Sr^{89} or Pu lived longer, on the average, than did similarly treated nonpregnant female mice. In addition, radiation damage was less severe among the Pu-treated mothers than it was among nonmothers.

3. The amount of Sr^{89} retained by the mothers immediately after parturition was the same as that retained by virgin adult females at comparable time intervals after injection. A portion of the material that would ordinarily have been excreted was lost through the placenta.

4. The percentage of the maternal dose of either Sr^{89} or Pu that was found in the young at birth varied with the injection-delivery interval. The Sr^{89} activity per gram of body weight of the newborn mouse exceeded that of the mother at the time of delivery if parturition occurred within the first 4 days after administration. At the levels studied, the concentration of Pu in the young never exceeded 8 per cent of the concentration in the mother.

5. Treatment of the young animals was continued during the period of suckling by the transfer of both Pu and Sr^{89} through the breast milk. Special studies of the transmission through this route of Sr^{89} were conducted by exchanging newborn litters of treated and normal mothers.

6. The initial excretion of Sr^{89} by very young mice is considerably lower than is the initial excretion by adults.

7. The animals which were treated *in utero* and before weaning with Sr^{89} have shown retardation of growth, malformation of the long bones, anemia, and osteogenic sarcoma. The animals of the Pu series have developed no pathologic conditions to date. There is no indication that fetal and very young tissues are either more or less sensitive to Sr^{89} and to Pu than are adult tissues.

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PLATE I

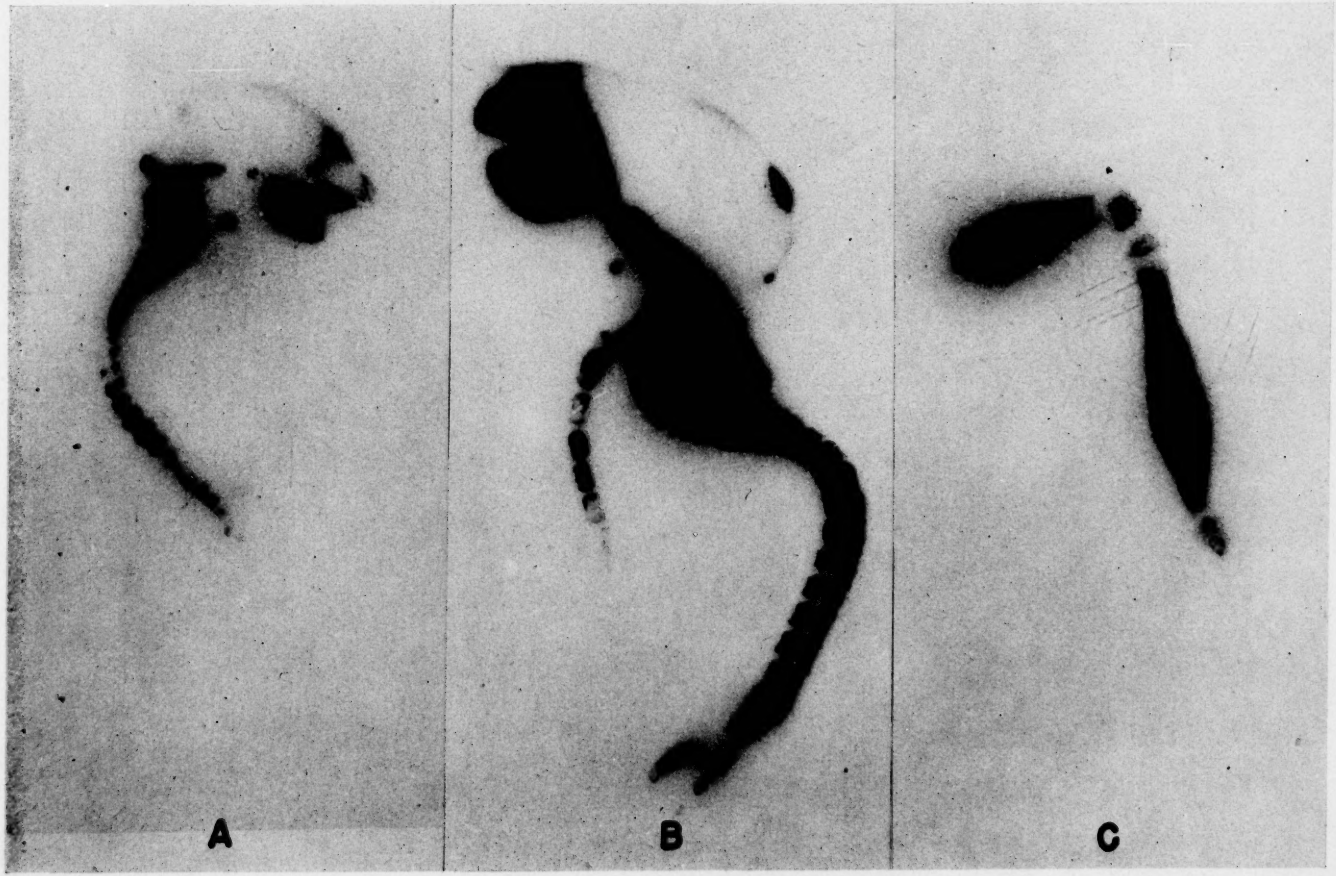

PLATE II

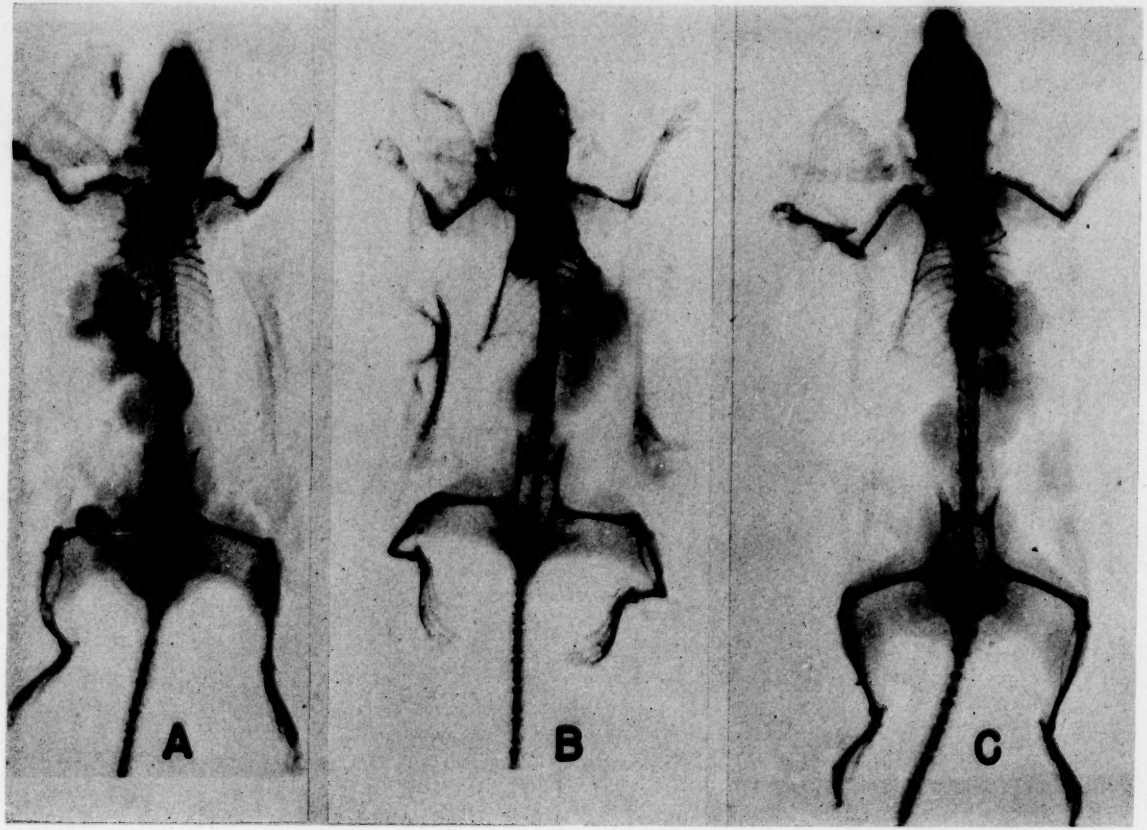

PLATE I

FIG. A.—Radio-autograph of mouse No. 2-12-81 at birth. Total Sr^{89} content = 0.17 μc. ×3.9.

FIG. B.—Radio-autograph of mouse No. 1-7-49 at 10 days of age. Total Sr^{89} content at birth = 6.9 μc. ×3.8.

FIG. C.—Radio-autograph of tibia of mouse No. 1-1-7 at 16 days of age. Total estimated Sr^{89} at 15 days = 10.6 μc. ×3.9.

PLATE II

FIG. A.—Roentgenogram of male mouse No. 2-3-22 at 325 days of age. Total estimated Sr^{89} at 15 days = 4.9 μc. Note malformed humeri and thickened, bent left tibia. Actual size.

FIG. B.—Roentgenogram of female mouse No. 2-3-25 at 309 days of age. Total estimated Sr^{89} at 15 days = 4.9 μc. Note bent tibiae. Actual size.

FIG. C.—Roentgenogram of control female mouse No. 4-12-81 at 295 days of age. Actual size.

NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

ANAEROBIOSIS IN INVERTEBRATES[1] BY THEODOR VON BRAND

Reviewed by WILLIAM BALAMUTH
Northwestern University

WRITING from an extensive research background in anaerobic metabolism, Von Brand has organized available data on anaerobiosis in invertebrates into a logical and solid frame of reference, which may well serve as a guide in suggesting and orienting much future work. The author has adopted a consistently biological approach and has directed the present work especially at ecologists and comparative physiologists, although all students of the invertebrates, parasitologists, and biochemists will profit as well.

The book comprises No. 4 of the "Biodynamica Monographs." It combines in large part the contents of reviews appearing in three recent numbers of *Biodynamica* and represents an extensive revision of a German review written by Von Brand in 1934.

The review is divided into three main sections: (1) the occurrence of anaerobiosis among invertebrates (128 pp.); (2) the anaerobic metabolism of invertebrates (120 pp.); and (3) general adaptation to the lack of oxygen and the origin of anaerobiosis (30 pp.). In the first section, following an outline and evaluation of general methods of investigation, the principal anaerobic habitats are discussed and are followed by a phyletic survey of the invertebrates for evidences of anaerobic habits. The habitats treated include soils, water basins, the intestinal tract, and tissues and secretions, so that considerable attention is devoted to animal parasites where these occur among Protozoa, Coelenterata, worms, Echinodermata, Mollusca, and Arthropoda. One point which emerges with special clarity concerns the futility of the debate over the aerobic versus anaerobic respiration of parasitic worms. The special organization of given parasites, and not the nature of the habitat, is apparently crucial in determining the predominant type of metabolism.

In the middle section of the review emphasis is shifted to some dynamic aspects of anaerobic metabolism: reversible transitions between aerobic and anaerobic metabolism, the occurrence of fermentations under aerobic conditions, the sources of energy drawn upon, and the processes controlling anaerobic functions. Normally aerobic forms often adopt an anaerobic metabolism under the influence of a lowered external oxygen tension. Evidences of this shift appear through identification of end-products of anaerobic metabolism, increase in rate of carbohydrate consumption and respiratory quotient, and temporary accumulation of an oxygen debt. Selected respiratory poisons and other experimental means can substitute for a changing oxygen tension in inducing this tran-

[1] Normandy, Mo.: Biodynamica, 1946. Pp. 328. $4.80.

sition. Aerobic fermentations (that is, incomplete oxidations in the presence of, but not utilizing, molecular oxygen) play an important supporting role in many Protozoa, worms, and arthropods; the most reliable criterion for their occurrence is the formation of incompletely oxidized substances in the presence of excess oxygen. Under anaerobic conditions some aerobic fermenters apparently obtain energy directly from the synthesis of fat, which is in direct contrast to the situation in most animals but which in this case is obviously of great adaptive advantage. As in vertebrates, carbohydrates (especially glycogen) are the most usual substrate for anaerobic energy. Isolated experiments suggest a fertile field for studying the role of lower carbohydrates, starch, cellulose, fats, and proteins, all of which have been reported to be sources of energy in specific invertebrates. In addition to lactic acid, fatty acids are produced, and various mixed fermentations occur in different groups. Carbon dioxide and combustible gases (for example, hydrogen) also have been reported. Few data are available as yet on anaerobic enzymes in invertebrates, but this is a promising field in view of the varied metabolic processes which have been described.

The concluding section of the monograph singles out for consideration such general biological problems as evidences of the basic adaptations permitting life under anaerobic conditions, the mode of origin of anaerobiosis, and the possible derivation of endoparasitism from anaerobic forms. The author can do little more than pose these problems, owing to the fragmentary state of knowledge in these areas. The recent work of Laser on *Ascaris* is cited to suggest that the injurious effect of oxygen on many anaerobes is due to low catalase content in these forms and consequent accumulation of toxic hydrogen peroxide. In those anaerobes surviving in the presence of oxygen an important adaptation consists of detoxifying processes or simply excretion of toxic end-products. An intriguing field concerns the possible role of environmental oxidation-reduction potentials in conditioning essential metabolic processes. From the few data available and by analogy with the extensive studies on bacteria, it appears that conditions producing low (negative) potentials encourage anaerobic growth. Both aerobic and anaerobic metabolism seem to have become well established before animal life evolved, although some evidence suggests that anaerobic metabolism is the more primitive type among ancestral forms.

Von Brand deserves considerable credit for organizing many diverse and often controversial data, extending into both foreign and American literature. His difficulty was not lessened by the questionable quality of much of the work, and it is to his credit that some attempt was made to evaluate some of it. It seems inevitable, however, that future studies employing modern techniques will render obsolete many of the data reviewed here. There are useful author and subject indexes and an extensive bibliography. A tabular appendix listing the faunas of various free-living anaerobic habitats nicely supplements the extensive tables in the first section, which summarize the literature dealing with survival time of different groups of free-living and parasitic invertebrates under anaerobic conditions.

Possibly the greatest single contribution of this work is to re-emphasize how incompletely explored are the basic fields of comparative physiology and comparative biochemistry. When considerably more body has been added to the general framework presented here, a great step

will have been taken in filling part of the relative void between cellular and mammalian physiology. Special opportunities are afforded by physiological researches on free-living and parasitic invertebrates: the animals concerned are favorable objects of study; the respiratory processes are more diversified than among vertebrates; and valuable information should be forthcoming as to the evolutionary origin of respiratory mechanisms in vertebrates.

CHEMISTRY OF MUSCULAR CONTRACTION[1] BY A. SZENT-GYÖRGYI

Reviewed by C. LADD PROSSER
University of Illinois

DURING the recent war years, while most biochemists and physiologists of combatant nations were sidetracked into applied science, Szent-Györgyi and his associates at Szeged and Budapest were engaged in brilliant research on the contractile proteins of muscle. The results of their experiments are presented in this book in greater completeness than in the summaries published previously (*Acta phys. skand.*, Vol. 9, Suppl. 25, 1945; *Jour. Coll. Sci.*, 1:1, 1946). The major part of the book is expanded from a lecture given at Edinburgh in July, 1946. Throughout the book a wise perspective toward the role of biochemistry in biology is maintained.

The first part of the book summarizes the properties of "myosin," and the second considers its function in muscle. Szent-Györgyi's theory of muscular contraction accounts for more properties of muscle than previous theories do and is based upon such close similarity between reactions of protein threads and of muscle that a revision of our thinking about contractile processes is necessary. In brief, the "old myosin" consists of two proteins: (1) myosin, an elongate molecule of molecular weight about 1,000,000 showing high viscosity and double refraction in water solution, precipitated by KCl in a narrow concentration range, and capable of adsorbing K^+ Mg^{++}, and Ca^{++}; (2) actin, a globular (G-actin) protein (probable m.w. 70,000), which can polymerize to a large fibrous (F-actin) form under the action of various salts. Mixture of myosin and actin (2.5 parts to 1) results in formation of actomyosin, which can be prepared in long threads. Precipitation (contraction of the threads) can be caused by KCl, but this precipitation is greater and in a narrower KCl range in the presence of adenosine triphosphate (ATP). In resting muscle the ATP is bound to actomyosin (AM) in an inactive state. The AM thread is pictured as a long double rod of myosin on one side and actin on the other; this is a hydrated, energy-rich state. By some excitatory process, such, possibly, as tearing off a K-ion, removal of ATP starts and continues autocatalytically. The free ATP then induces a molecular rearrangement of myosin such that it shrinks and loses intermicellar water. The actin splits into globules, so that the whole rod curls, and the actomyosin is now energy poor. Experiments on ATP-initiated shortening of the threads at different temperatures show that the

[1] New York: Academic Press, 1947. Pp. vi+150 +49 figs. $4.50

process is endothermic and that at 37° the free energy decreases by 7,000 calories. In the presence of Mg^{++} and Ca^{++} the energy-poor myosin enzymatically splits ATP, liberating 11,000 calories for each phosphate bond broken; this provides energy for relaxation.

> F-actomyosin is brought to contraction going over into the energy-poor dehydrated form. At the same time actin breaks up into globules. By going over into the contracted state the actomyosin becomes enzymatically active, splits ATP, going back into the energy-rich hydrated form. Under the action of ATP this actomyosin dissociates into free G-actin and free myosin. The G-actin then polymerizes into F-actin; we return to our starting point and the system is ready for a new contraction. . . . . The energy of fermentation is made available to the contracting mechanism in the form of the phosphate bonds of ATP. If the muscle needs energy it splits these bonds and the dephosphorylated ATP sets the mechanism of fermentation going by serving as phosphate acceptor.

Creatine serves the role of phosphate carrier.

In Part III, Szent-Györgyi discusses his "continuum theory" of the nature of living matter. He presents evidence for band spectra in protein aggregations. That is, the valence electrons may be shared by different molecules, and proteins may form a sort of energy continuum, along which energy (electrons) may travel a certain distance through the system of many molecules. A cell wall may be the border line of common energy levels.

Part IV presents trends in current research (1947). One important development is the splitting-off from the skeleton of myosin of several components called "protins" (prosthetic proteins). Some of these are distinguished by their enzymatic properties, one as a chromoproteid. In addition, myosin has a lipid component, probably a cerebroside.

A second important trend in current research concerns the nature of the striations in muscle. Earlier attempts by Szent-Györgyi to account for striations on the basis of spiraling of the actomyosin fibrils are not entirely satisfactory. It is surprising that the striations remain after most of the myosin has been extracted; extraction of actin also left the striations, but the double refraction became negative, the bands reversing.

Part V presents methods of separation and purification of ATP, myosin, actin, and actomyosin threads.

This book is a progress report. Such concepts as that energy from ATP is used in relaxation and that the striations are independent of myosin are so important that they need extensive checking. The total picture is, however, brilliantly conceived and well executed. The weakest points in the theory appear to be (1) the lack of information regarding the mechanism which starts the cycle by removing ATP; (2) absence of a precise picture of the rearrangement in the myosin molecule as it shrinks and becomes dehydrated; and (3) inconclusive experiments on the energy relations. The history of muscle physiology is marked by frequent "revolutions"; the current work of Szent-Györgyi and others on muscle proteins represents a new and vigorous uprooting of classical ideas.

INDEX TO VOLUME XX

Actions of Radiations on Living Cells by D. E. Lea, 322
ALLEE, W. C., 110
Anaerobiosis in Invertebrates by Theodor von Brand, 422
Analysis of the Factors Influencing Growth and Development of the Mammalian Reproductive Tract, An, 213
Analysis of Photoelectric Instruments for Measurement of Turbidity with Reference to Serology, An, 172
ANDREWS, E. A., 1
Animal Cytology and Evolution by M. J. D. White, 210

BAIER, JOSEPH G., JR., 172
BALAMUTH, WILLIAM, 422
BARTH, L. G., 111, 133
BEEMAN, ELIZABETH A., 373
BENNETT, GEORGE W., 349
Biological Actions of Sex Hormones by Harold Burrows, 108
Biological Basis of Individuality, The by Leo Loeb, 114
BLIVAISS, BEN B., 67
BODINE, JOSEPH HALL, 146
BOWEN, W. J., 165
BRACHET, JEAN, 111
BRAND, THEODOR VON, 422
BURROWS, HAROLD, 108

Chemical Senses, The by R. W. Moncrieff, 209
Chemistry of Muscular Contraction by A. Szent-Györgyi, 424
CHILD, C. M., 332

Directiveness of Organic Activities, The by E. S. Russell, 110

Effect of Certain Deficient Media on Resting Cyst Formation in *Colpoda duodenaria*, 5
Effect of Certain Denatured Organs on Neuralization in *Amblystoma* Explants, The, 32
Effect of Certain Depressing Agents on Intracellular Oxidation of Indicators in Hydra, The, 332
Effect of Male Hormone on Aggressive Behavior in Mice, The, 373
Effect of Salts and Osmotic Concentration on the Size of Food Vacuoles in *Vorticella similis*, The, 165
Effect of Temperature on the Intersex Character of *Daphnia longispina*, The, 325
Effect of Thiourea on the Oxygen Consumption of *Fundulus*, The, 161
Effects of Testosterone Propionate on Sex Development in Female *Ambystoma* Larvae, 355
Embryologie chimique by Jean Brachet, 111

FINKEL, MIRIAM P., 405
FITZGERALD, LAURENCE R., 146

GARNJOBST, LAURA, 5
GERARD, R. W., 114
GREENBERG, BERNARD, 267

HESS, CARL W., 125

Influence of the Sinus Glands upon Molting and Associated Changes in the Crayfish, The, 187
Interrelations of Thyroid and Gonad in the Development of Plumage and Other Sex Characters in Brown Leghorn Roosters, 67

JAEGER, L., 133
JAHN, THEODORE LOUIS, 209
JAMES, MARIAN F., 349
JUHN, MARY, 117, 125

LEA, D. E., 322
LIMBACH, BRUNO VON, 349
LOEB, LEO, 114

MATTHEWS, SAMUEL A., 161
MINTZ, BEATRICE, 355
MONCRIEFF, R. W., 209
MOORE, CARL R., 300

New books in physiological zoölogy, 108–15, 209–11, 322–24, 422–25
Notice to contributors, 212, 324

Origin of Pigment Cells from the Neural Crest in the Mouse Embryo, 248
ORTIZ, EVELINA, 45
Ovarian Activity in Mammals Subsequent to Chemical Injury of Cortex, 300

Phosphorylation in the Frog's Egg, 133
Plumage Sequence in a Case of Hemilateral Asymmetry in Male Hybrid Fowl, 117
Postnatal Development of the Reproductive System of the Golden Hamster (*Cricetus auratus*) and Its Reactivity to Hormones, 45
PRICE, DOROTHY, 213
PROSSER, C. LADD, 349, 424

RAWLES, MARY E., 248
Relation of the Plumage-Tract Gradients to the Serial Localization of Feather Pigments in a Hybrid Cock, The, 125
Responses of Fish Melanophores to Chemical Warfare Agents, 349
Riboflavin and Other Fluorescent Compounds in a Developing Egg (Orthoptera), 146
RUSSELL, E. S., 110

SANFORD, KATHERINE K., 325
SCUDAMORE, HAROLD H., 187
SHEER, BRADLEY T., 15
SHEER, MARLIN ANN RAY, 15
SMITH, DIETRICH C., 161
Some Interrelations of Drug and Ion Actions in the Artificial Activation of Marine Eggs, 15
Some Relations between Territory, Social Hierarchy, and Leadership in the Green Sunfish (*Lepomis cyanellus*), 267
SZENT-GYÖRGYI, A., 424
Temperature Effect upon Rate of Feeding in a Folliculinid, 1
Transmission of Radio-Strontium and Plutonium from Mother to Offspring in Laboratory Animals, The, 405
TURNER, C. DONNELL, 108
TURNER, C. L., 210
VAN CLEAVE, C. D., 32
WANG, HSI, 300
WHITE, M. J. D., 210
ZIRKLE, RAYMOND E., 322

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Volume XX Number 4

Physiological Zoölogy

OCTOBER 1947

The Effect of Temperature on the Intersex Character of *Daphnia longispina*
Katherine K. Sanford 325

The Effects of Certain Depressing Agents on Intracellular Oxidation of Indicators in Hydra . . . . . *C. M. Child* 332

Responses of Fish Melanophores to Chemical Warfare Agents
C. Ladd Prosser, Bruno von Limbach, George W. Bennett, and Marion F. James 349

Effects of Testosterone Propionate on Sex Development in Female *Ambystoma* Larvae . . . . . *Beatrice Mintz* 355

The Effect of Male Hormone on Aggressive Behavior in Mice *Elizabeth A. Berman* 373

The Transmission of Radio-Strontium and Plutonium from Mother to Offspring in Laboratory Animals . . . . . *Miriam P. Finkel* 405

New Books in Physiological Zoölogy 422
Theodor von Brand, Anaerobiosis in Invertebrates, *William Bahnemann*.—A. Szent-Györgyi, Chemistry of Muscular Contraction, *C. Ladd Prosser*

Index 427

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